

Thesis for the Master's degree in Molecular Biosciences  
Main field of study in Molecular Biology

# **Expression of the bacterial *ectB* gene in the chloroplast of *Chlamydomonas reinhardtii***

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60 study points

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## Summary

Ectoine is a small compound synthesized in the cytoplasm of halophilic and halotolerant bacteria in response to salt stress. The compound has been found to behave as a compatible solute counterweighing osmotic imbalance between the interior and exterior of a cell. Because of its positive effects on the water balance of cells and its protective properties ectoine isolated from bacteria is broadly used in medical therapies and biotechnology, e.g. as an additive in cosmetics.

The goal of this study was to synthesize ectoine in the chloroplast of the unicellular green alga *Chlamydomonas reinhardtii* in order to increase the cells' salt tolerance. In bacteria synthesis of ectoine is catalyzed by proteins encoded by the *ectA*, *ectB* and *ectC* genes. As the *ectC* gene was thought to be dispensable for ectoine synthesis in *Chlamydomonas*, only the bacterial *ectB* and *ectA* genes were stably inserted by biolistic transformation into the chloroplast genome of the alga. The resulting transgenic cell lines were investigated and analysed by DNA and RNA blotting techniques for the presence of the genes in the chloroplast genome, for accumulation of *ectA* and *ectB* transcripts, and for improvements in salt tolerance due to ectoine accumulation.

The results showed that the introduced *ectA* and *ectB* genes were expressed in *C. reinhardtii* but no improvements in salt tolerance of the transformants could be detected. It is concluded that expression of *ectA* and *ectB* in the chloroplast of *C. reinhardtii* is not sufficient for ectoine accumulation. It is suggested that further work focuses on additional expression of the *ectC* gene and on codon optimization of *ectA* and *ectB* genes.

## Abbreviations

approx.	Approximately
ATP	Adenosine-5'-triphosphate
<i>atpB</i>	Coding for ATP synthase complex, subunit $\beta$
bp	Base pair
BSA	Bovine serum albumin
Ca.	Circa
CIP	Calf Intestinal Alkaline Phosphatase
cm	Centimetre
DEPC	Diethylpyrocarbonate
dH <sub>2</sub> O	Distilled H <sub>2</sub> O
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycolaacetic acid
g	Gram
g	Gravitational force
kb	Kilobase pair
L	Litre
M	Molar concentration
mA	Milliampère
mg	Milligram
min.	Minutes
mL	Milliliter
mm	Millimeter
mM	Millimolar concentration
MOPS	3-morpholinopropane-1-sulfonic acid
mRNA	Messenger RNA
N	Normality
NEB	New England Biolabs
ng	Nanogram
nm	Nanometer
OD	Optical density
PCR	Polymarese chain reaction
PEG	Polyethylene glycol
pmol	picomol
<i>psaB</i>	Photosystem I subunit B
<i>psbA</i>	Coding for Photosystem II subunit DI
<i>psbD</i>	Coding for Photosystem I subunit D

<i>rbcL</i>	Ribulose Biphosphate Carboxylase large subunit
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxidative species
s	Second
SDS	Sodium deodecyl sulphate
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
u	Units
UTR	Untranslated regions
UV-light	Ultraviolet light
V	Volt
w/v	Weight by volume
w/w	Weight by weight
μCi	Microcurie
μg	Microgram
μL	Microliter
°C	Degrees celsius
x	Times

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# 1. INTRODUCTION

## 1.1 Challenging salinity

### 1.1.1 Organisms in hypersaline environments

Most living organisms have difficulties growing and thriving in a hypersaline environment. High extracellular salt concentrations result in water diffusing out of the cell due to high outside water potential. Depending on the level of salt tolerance, such unfavourable settings may lead to loss of cellular water eventually causing cell death (Mahajan & Tuteja, 2005).

Organisms living in extreme conditions are called extremophiles. They have through evolution developed several properties to tolerate and thrive in extreme habitats.

Extremophiles living in environments with high salt concentrations are classified as either halophiles or halotolerants. Halophiles and halotolerants are found in various habitats, often within salt lakes, salt marshes, springs and other sites with elevated salt concentrations. Most halophiles and halotolerants are archaeal, while others belong to the kingdom of bacteria or eukaryota (Fukuchi et al., 2003; Oren, 1999). Halophiles require salt for survival and have optimal growth at concentrations of up to 0.85 M NaCl, tolerating at least 1.7 M NaCl. In contrast, halotolerants do not require any specific salt concentration for growth and tend to tolerate a wide range of salt concentrations (Oren, 2008).

As a rule, proteins in halophiles are associated with increased salt tolerance. A number of studies have shown that extreme halophiles often have small proteins with short side chains, acidic amino acids and non-hydrophobic residues. Thus, tightly packed proteins are essential for maintaining the protein conformation on exposure to high salt concentrations (Fukuchi et al., 2003; Lanyi, 1974; Tadeo et al., 2009). These characteristics are even harmful for certain extreme halophiles when introduced to an environment with lower salt concentration. The proteins are inactivated and the cell wall destabilized as a result of difficulties associated with adaption to lower salt concentrations (Lanyi, 1974; Oren, 2008).

### **1.1.2 Salt surviving strategies**

$\text{Ca}^{2+}$  is well known for taking part in alleviating the detrimental effects caused by excessive salt in cells (Mahajan & Tuteja, 2005). Apart from  $\text{Ca}^{2+}$  interference, halophiles and halotolerants have evolved two different strategies in order to counteract problems related to saline conditions.

The first strategy maintains the water levels in the cell by accumulating inorganic compounds intracellularly, mainly by influx of KCl and efflux of  $\text{Na}^+$  catalyzed by corresponding ion transporters. Hence, osmotic imbalance is avoided due to similar internal and external salt concentrations. A few extremely halophilic archaea and anaerobic moderately halophilic bacteria of the order Haloanaerobiales have adopted this method. Organisms committed to this strategy have highly acidic proteomes that will denature when moved from high to low salt concentrations (Oren, 1999).

An alternative strategy applied against water loss is the usage of compatible solutes, which are acquired by the majority of halophilic bacteria. Under hypersaline conditions, compatible solutes are synthesized and accumulated within the organism. Their purpose is to maintain proper osmotic balance by excluding salt from the cell's cytoplasm, and creating a low osmotic potential inside the cell (Louis & Galinski, 1997), thereby preventing water loss and increasing salt tolerance. This strategy is rather flexible unlike the strategy based on intracellular accumulation of salt. Flexibility is due to swift adjustment in concentration of intracellular compatible solutes in response to changes in the extracellular environment. Synthesis of organic solutes is a more energy requiring process compared to accumulation of inorganic compounds (Oren, 1999).

### **1.1.3 Compatible solutes**

The term compatible solute was first used by Brown and Simpson (1972), but the definition has since changed. Nowadays compatible solutes are signified as small and soluble organic compounds, who maintain osmotic balance between the extracellular environment and the cytoplasm. They manage to do this while being “compatible” with the organism's biomolecules, implying they do not disrupt vital cellular functions, metabolic processes or protein folding (Brown, 1990; Oren, 1999). Compatible solutes are also called osmolytes or

osmoprotectants, and are found among all the three domains of life (Roberts, 2005). Compounds classified as compatible solutes include: amino acids such as proline and glutamic acid, their derivatives (glycine betaine, ectoine and hydroxyectoine), some sugars and polyols (Galinski, 1995).

#### **1.1.4 Production of compatible solutes by genetically engineered crops**

On a world basis salt stress causes major detrimental effects on crop growth and productivity. Hence it is important to understand the molecular basis for salinity tolerance to improve agricultural growth. There has been a considerable effort from the scientific community to design salt tolerant transgenic crops. A number of studies have described elevated salt tolerance in genetically engineered crops by using genes that encode compatible solutes (Moghaieb et al., 2011; Nakayama et al., 2000).

So far, attempts to develop transgenes have mostly been done with model organisms such as *Arabidopsis* and tobacco plants (Hayashi et al., 1998; Moghaieb et al., 2006). Even though introducing salt tolerance traits is complicated and involves multiple genes (Flowers, 2004), such genes have successfully been introduced into plants providing knowledge about metabolites and techniques. However, engineering of salt tolerance into plants has only been partially successful.

According to Chen et al. (2002), most of the genetically engineered plants synthesize low levels of compatible solutes, with quantities that are not high enough to execute a proper osmotic balance. A slight increase in the tolerance of stress might be a result of other features of compatible solutes, such as protein protection activities and reduction of ROS (reactive oxidative species) (Chen et al., 2002).

Moreover, the idea of transferring bacterial salt tolerance genes to a plant may create mRNA translation difficulties due to different codon usage between the species. Another impediment is the energetically expensive production of compatible solutes, which thereby affects energy resources and crop yield. Associated complications are the ability of various crops to synthesize and accumulate different types of compatible solutes, which is somehow limited (Rontein et al., 2002).

## 1.2 Ectoine

### 1.2.1 Ectoine as a compatible solute

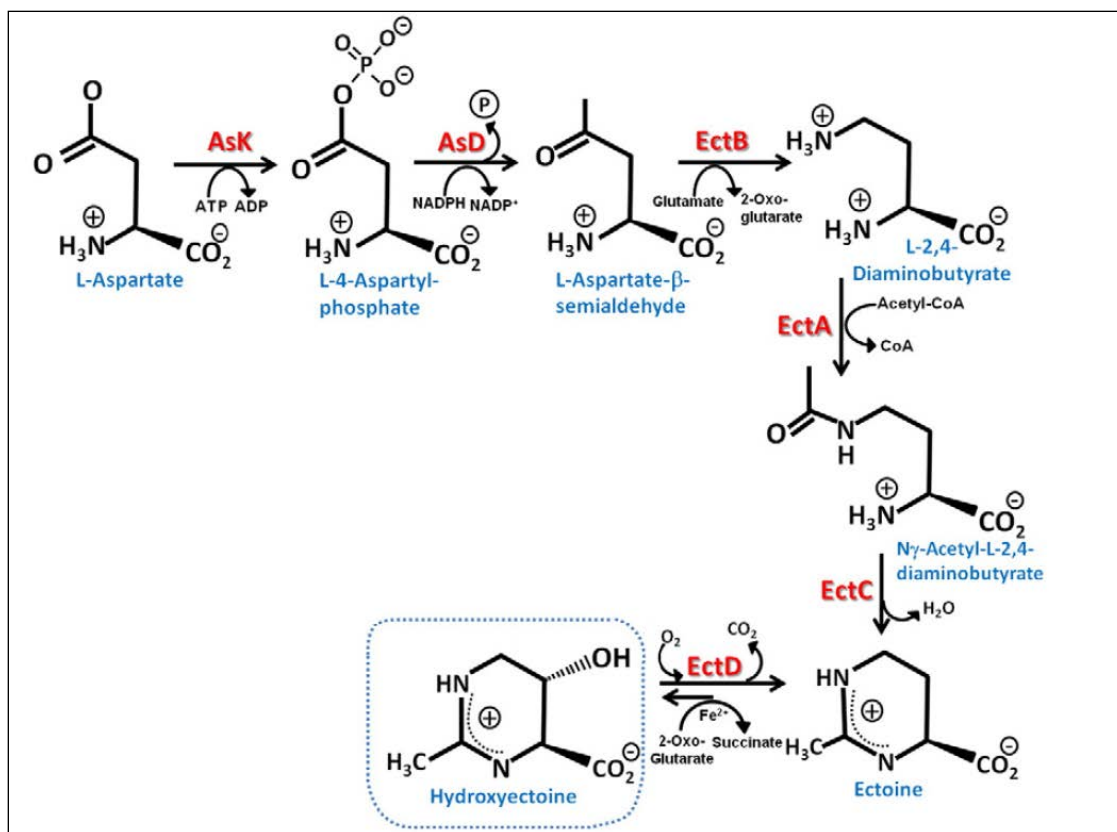
The most abundant compatible solute is ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid), an uncharged or zwitterionic compound, which was originally discovered in the phototroph *Halorhodospira halochloris* by Galinski et al. (1985). Ectoine is commonly found in aerobic chemoheterotrophic bacteria (Galinski, 1995). Under increased extracellular salt levels, this cyclic compound is synthesized and accumulated in the cytoplasm or taken up from a culture medium if available.

### 1.2.2 The biosynthesis of ectoine

The biosynthetic pathway of ectoine includes 3 major steps catalyzed by L-2,4-diaminobutyric acid transaminase, L-2,4-diaminobutyric acid acetyltransferase and L-ectoine synthase. These proteins are encoded by the *ectB*, *ectA* and *ectC* genes, respectively (Figure 1.1).

The biosynthesis begins with the conversion of aspartate- $\beta$ -semialdehyde to L-2,4-diaminobutyrate by L-2,4-diaminobutyric acid transaminase (*ectB*). L-2,4-diaminobutyric acid is acetylated by L-2,4-diaminobutyric acid acetyltransferase (*ectA*) to form N <sup>$\gamma$</sup> -acetyl-L-2,4-diaminobutyric acid. Further on, NaCl activates ectoine synthase (*ectC*), which converts N <sup>$\gamma$</sup> -acetyl-L-2,4-diaminobutyric acid to ectoine. *EctABC* genes are transcribed from one promoter as a single operon.

During the unravelling of this biosynthetic pathway, the *ectABC* gene cluster involved was isolated from *Marinococcus halophilus* (Louise & Galinski, 1997). A number of organisms that harbour *ectABC* genes synthesize in addition the ectoine derivate hydroxyectoine (Seip et al., 2011).



**Figure 1.1:** The biosynthetic pathway of ectoine and hydroxyectoine. Aspartate derived from the amino acid biosynthetic pathway takes part in ectoine synthesis. The figure is a slightly modified version of a figure from Pastor et al. (2010).

### 1.2.3 Supporting cell protection and protein stability

Compatible solutes are termed “chemical chaperones” due to their stabilization traits. There have been several reports on compatible solutes, including ectoine and hydroxyectoine, participating in preservation against protein misfolding and stabilizing proteins (Graf et al., 2008; Knapp et al., 1999).

The presence of certain compatible solutes initiates an interaction between protein and solute, which exclude the solute from the protein surface due to hydration. Simultaneously, the protein is forced into its native form, which is compact and occupies less space (Liu & Bolen, 1995). In 1999, Göller and Galinski presented a similar model for protein stabilization by ectoine, which supported a previous study on compatible solutes (Arakawa & Timasheff, 1985).

Ectoine has in addition been shown to be a compound that stabilizes nucleic acids and gives cell protection against stresses like freezing, drought and heat. It has proven to be the most stabilizing compound among compatible solutes (Lippert & Galinski, 1992). Besides, ectoine and hydroxyectoine are able to alter the conformation of DNA in such way that endonucleases can not cleave it (Malin et al., 1999).

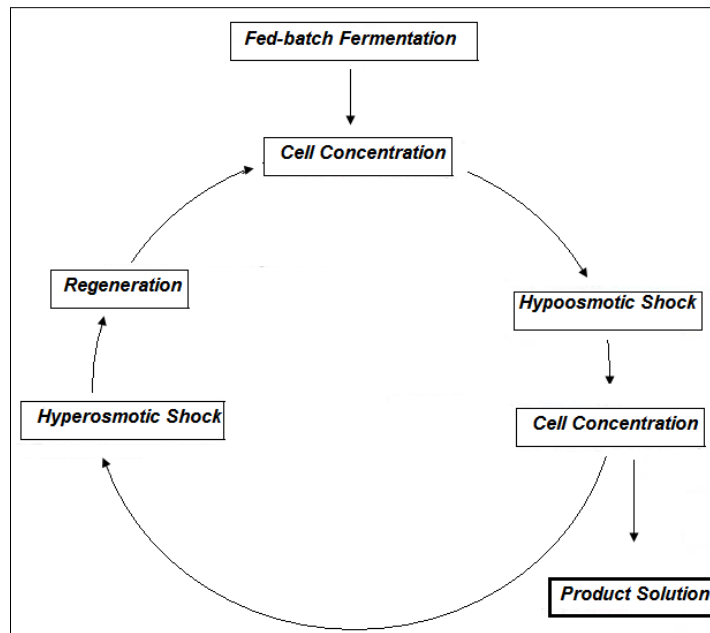
#### **1.2.4 Application of ectoine**

Ectoine has multiple advantageous functions and thereof many applications. Foremost is its ability in cosmetics as a skin moisturizer, where it works as an osmolyte. It reduces stress factors that lead to aged skin, as documented by Heinrich and colleagues (2007). Likewise, ectoine is also used in sunscreen creams because of its capability to protect skin from UVA-rays (Buenger & Driller, 2004).

Furthermore, it maintains protein folding and protects proteins from proteolysis. This may be utilized against diseases associated with protein misfolding and generally in development of pharmaceuticals (Kolp et al., 2006). Regarding other biotechnological applications, ectoine has a potential to improve both crop productivity and quality, though a better understanding of ectoine synthesis and its related cellular pathways is needed.

#### **1.2.5 Industrial production of ectoine**

The numerous applications of ectoine, especially as an additive in cosmetics, require an industry-type ectoine production to meet an increasing demand. To produce ectoine in large-scale, fermentation procedures such as “bacterial milking” are frequently used for extracting metabolites (Sauer & Galinski, 1998). The bacteria are placed into a medium with high salinity in which they produce compatible solutes (hyperosmotic shock), a sudden transfer to a medium with lower salt concentration (hypoosmotic shock) results in release of compatible solutes. Switching between osmotic shocks in combination with filtration techniques is used to harvest the compatible solutes (Figure 1.2). In order to solely obtain ectoine, it has to be isolated and purified from among other compatible solutes present.



**Figure 1.2:** The bacterial milking technique for production of compatible solutes in *H. elongata* used by Sauer and Galinski (1998). The flow diagram demonstrates how ectoine is made by repeatedly undergoing osmotic changes and filtration steps, before they are isolated and purified. The figure presented is from Galinski and Louis (1998).

The bacterium *Halomonas elongata* is usually chosen as the ectoine production factory, mainly because of its ability to grow in a wide range of salt concentrations. Secondly, due to its rapid response in synthesizing and releasing ectoine through transporters (Fischel & Oren, 1993; Sauer & Galinski, 1998).

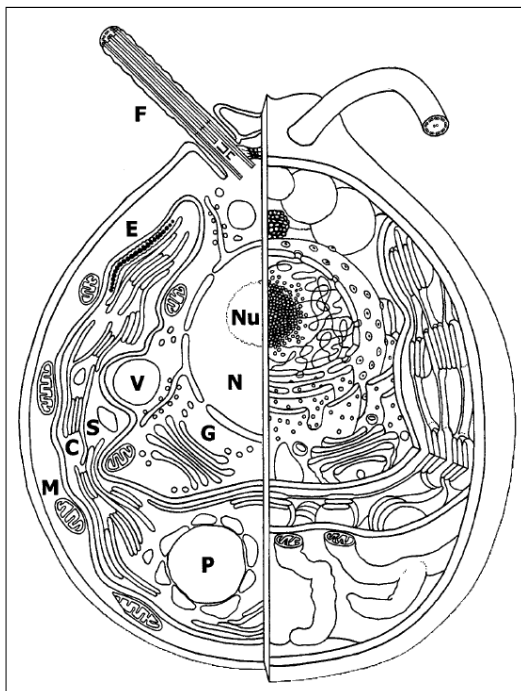
New techniques and species are under investigation for improvement of production and purification of various compatible solutes (Becker et al., 2013). A recent report demonstrated how different types of sugars available to engineered *H. elongata* may affect production rates of ectoine (Tanimura et al., 2013). Results showed that *H. elongata* produced ectoine more efficiently with biomass-derived xylose, than with glucose that had been commonly used in previous studies.

## 1.3 *Chlamydomonas reinhardtii*

### 1.3.1 Physiology of *Chlamydomonas reinhardtii*

*Chlamydomonas reinhardtii* (hereafter called *Chlamydomonas*) is a haploid photosynthetic eukaryotic green alga. It is widely distributed over the world and can be found in different habitats, especially in soil and fresh water. The wild-type *C. reinhardtii* strain utilized in laboratories was originally isolated in the 1940s from soil in Massachusetts (US) by Gilbert M. Smith (Harris, 2001).

The morphology of *Chlamydomonas* is quite prominent: an oval shaped cell body of 10  $\mu\text{m}$  in diameter with two flagella extending from the anterior end (Figure 1.3). Unlike the cell wall of land plants, the *Chlamydomonas* cell wall consists of seven layers of hydroxyproline-rich glycoproteins surrounding the cell content. Additionally it contains a pyrenoid within the chloroplast, mitochondria, a Golgi apparatus near the nucleus, and two contractile vacuoles at the anterior end involved in osmoregulation. The two flagella help the microalgae orientate according to its light sensing organ, the red pigment eyespot. Thus it can adjust the amount of light during photosynthesis (Harris, 2001). Instead of phytochromes as photoreceptors, it has cryptochrome which is also found in higher plants and animals (Funes et al., 2007). Furthermore, the chloroplast genome of the *Chlamydomonas* and its photosynthetic apparatus has some similarities with both higher and lower plants.



**Figure 1.3:** Cell structure of *Chlamydomonas reinhardtii*. Nucleolus (Nu), nucleus (N), golgi vesicle (G), vacuole (V), starch grain (S), chloroplast (C), mitochondrion (M), pyrenoid (P), eyespot (E) and flagella (F) (Nickelsen and Kück, 2000).



### **1.3.2 *Chlamydomonas reinhardtii* as a model organism**

*C. reinhardtii* has been used as a model organism particularly in studies of motility, chloroplast biogenesis, mitochondria and photosynthesis (Funes et al., 2007). Much of our current understanding of the chloroplast and the photosynthetic machinery has been achieved through research on *Chlamydomonas*. In contrast to higher plants, *C. reinhardtii* can grow heterotrophically in the dark with acetate, and photosynthetically with light as the only energy source. It has a short generation time (~6 hours) and can grow in simple media without any supplementary vitamins or co-factors (Harris, 2001).

All three genomes of the alga have been entirely sequenced; the mitochondrial genome with 15.8 kb (Vahrenholz et al., 1993) and the circular chloroplast genome which is approx. 200 kb (Maul et al., 2002). In 2007, Merchant et al. sequenced the nuclear genome that has a size of approx. 120 Mb.

Analyzing mutations in such organisms since their haploid nature makes mutations appear rapidly and easily. Their heterotrophic trait creates an opportunity to investigate mutants with no photosynthetic machinery (Funes et al., 2007). Besides, transformation techniques have been developed for all three genomes.

### **1.3.3 Foreign protein expression in the chloroplast of *Chlamydomonas***

During the past decades, recombinant proteins have gained much attention and are being researched on in various expression systems. There is great interest in the search for expression systems that are safe, inexpensive and have high yields (Mayfield et al., 2007).

There are several benefits in using the chloroplast of microalgae as a manufacturing system for foreign proteins (Table 1.1). First of all, plastid genes are dependent on their own translation machinery for RNA- and protein-synthesizing systems for gene expression. Secondly, transgenic microalgae can be grown axenically, free from external contamination and harmful effects. Furthermore a high yield of recombinant proteins can be obtained rapidly and inexpensively compared to isolation from traditionally cultured cells (Mayfield et al., 2007).

Recombinant proteins produced in <i>C. reinhardtii</i> chloroplasts.		
Protein product	Application/comments	Reference
Aminoglycoside adenine transferase	Reporter, confers spectinomycin and streptomycin resistance	[8]
$\beta$ -Glucuronidase	Reporter, catalyzes the conversion of substrates to colored products	[9]
Renilla luciferase	Reporter protein, luminescent	[10]
Aminoglycoside phosphotransferase	Reporter, confers kanamycin and amikacin resistance	[49]
Green fluorescent protein	Reporter protein, fluorescent	[13]
HSV8-lsc	Pharmaceutical, first mammalian protein expressed	[2**]
Cholera toxin B subunit fused to foot and mouth disease VP1	Pharmaceutical, vaccine	[36**]
Bacterial luciferase	Real-time reporter protein, can be visualized in living cells	[14*]
HSV8-scFv	Pharmaceutical, classic single-chain antibody	[50]
Allophycocyanin	Fluorescent protein	[48**]
Human metallothionein-2	Pharmaceutical, UV protection	[38]
Firefly luciferase	Real-time reporter protein, can be visualized in living cells	[51**]
Human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)	Pharmaceutical	[37]

**Table 1.1:** Recombinant protein expression in the chloroplast of *Chlamydomonas* (Mayfield et al., 2007).

A stable and high level gene expression in the chloroplast of *Chlamydomonas*, whether of natural origin or recombination, is dependent upon various criteria. Most importantly, codon optimization adjustments for the desired genes are required (Weiß et al., 2012). Chloroplast expression constructs having optimized codons have been shown to increase recombinant protein synthesis (Franklin et al., 2002). Such constructs consist of a strong promoter (Table 1.2), 5' UTR and 3' UTR. All the necessary regulatory elements for expression are present except the coding region of the protein, which would be flanked by the promoter and the terminator (Klein et al., 1994).

Promoter	Gene source	Highest expression level achieved (% TPS)	Construct (Prom-gene-3'UTR)	Strain (mt+)	Reference
atpA	Alpha subunit of adenosine triphosphatase	~3-4	atpA-CTBVP1-rbcL	137c	Sun et al. (2003)
psbD	Photosystem II D1	~0.25	psbD-M-SAA-psbA	137c	Manuell et al. (2007)
psbA	Photosystem II psbA	~5	psbA-M-SAA-psbA	137c	Manuell et al. (2007)
psbA	Photosystem II psbA	~20	psbA-VP28-psbA	741c	Surzycki et al. (2009)
psbA	Photosystem II psbA	~3	psbA-14FN3-psbA	137c	Rasala et al. (2010)
rbcL	Ribulose biphosphate carboxylase large subunit	~0.7	rbcL-CTBD2-rbcL	125c	Dreesen et al. (2010)

**Table 1.2:** Various chloroplast promoters along with corresponding constructs that have been used for protein expression. This table is a modified version of a table from Rosales-Mendoza et al. (2011).

In 2009, Surzycki et al. postulated that the expression of recombinant protein is more dependent upon the transformation event in the chloroplast, rather than on an optimal promoter and UTR. After a homologous recombination, identical transformants termed transformosomes had varying amount of protein accumulated in the chloroplast. This was explained to be a consequence of additional transgene insert events taking place in the nucleus and the chloroplast, which were influencing the protein expression that regulate recombinant yields.

### 1.3.4 Optimized codon usage

The genetic code consists of triplets that encode amino acids, and they collectively make up the proteins. Certain codons may appear more frequently in certain species, or in different intracellular genomes (Weiß et al., 2012). The chloroplast of *Chlamydomonas* has a strong codon bias towards AT in the third position, whereas the nuclear genome is usually rich in GC

(Merchant et al., 2007). Hence the translation efficiency is often low when expressing transgenic proteins and a common solution is codon optimizing to highly expressed proteins (Surzycki et al., 2009). Studies have been performed in order to test whether codon optimization improves the recombinant protein expression in *C. reinhardtii* chloroplasts (Wu et al., 2011). Franklin et al. (2002) transformed codon-optimized *gfp* (green fluorescent protein) and nonoptimized native *gfp* genes with the same promoter and UTR into the chloroplast. Results indicated that the codon-optimized *gfp* gene had an 80-fold increase in protein accumulation compared to the non-optimized one.

### **1.3.5 Transformation by microprojectile bombardment**

Microprojectile transformation is an effective method for transferring DNA to cells. It allows studying transgenes and the alga's ability to carry out expression of introduced genes (El-Sheekh, 2000). The first successful microprojectile bombardment of foreign DNA into the chloroplast of *Chlamydomonas* was accomplished by Blowers and colleagues (1989). The method is performed by shooting tungsten or gold-particles coated with DNA into the cell of interest. The high kinetic energy allows the DNA to penetrate not only the cell wall, but also the double membrane of chloroplasts. Further on, the DNA can be integrated by homologous recombination into the chloroplast genome if the elements for a possible recombination are present (El-Sheekh, 2000; Harris, 2001).

In homologous recombination the integrated DNA can be targeted precisely to a specific region in the chloroplast genome (Goldschmidt-Clermont, 1991). There are approximately 50-80 copies of the DNA chromosome in the chloroplast of a *Chlamydomonas* cell which may integrate the recombinant form. If all chloroplast genomes contain the inserted gene, the chloroplast is called homoplasmic, otherwise it is heteroplasmic (Rochaix, 1995). Chloroplast genomes lack disadvantages such as gene silencing, instability and epigenetic interference mechanisms, which make recombinant protein expression easier compared to the nucleus (Bock, 2007).

All three genomes of *Chlamydomonas* have been reported to have undergone successful transformation (Harris, 2001), either by microprojectile transformation, electroporation, or vortexing DNA and cells in the presence of glass beads.

### 1.3.6 Ectoine production in *Chlamydomonas reinhardtii*

Ectoine has been synthesized *de novo* in bacterial cells and plants, though with highest yield in bacteria. Nonetheless, the unicellular organism *Chlamydomonas* is an optional producer of ectoine with low salt requirements. *Chlamydomonas* is a valuable production host in biotechnology, primarily because its molecular biology is well understood due to studies as a model organism. Expressing ectoine in genetically modified *Chlamydomonas* may give insight into whether ectoine may be possible to synthesize in quantity in higher plants, such as crops. However, there has not been any ectoine production in *Chlamydomonas* chloroplast demonstrated so far.

The nuclear genome of higher plants has been transformed with *ectABC* genes (Moghaieb et al., 2011), but not the chloroplast genome. Gene expression in the chloroplast would give increased protein production, which would be beneficial for obtaining salt tolerant cells or bulk isolation of ectoine from *Chlamydomonas*. Additionally, the precursor of ectoine, aspartate- $\beta$ -semialdehyde, is probably present in higher amount in chloroplasts than in the cytoplasm due to their high rates of amino acid synthesis (Louis & Galinski, 1997).

Achieving high expression levels of foreign genes has been challenging (Lunde, 2012). Lunde (2012) didn't observe any expression of EctA, nor was there any production of ectoine in the *C. reinhardtii* transformant to improve salt tolerance. In order to produce ectoine, the *ectA* gene alone is not sufficient. The *ectB* gene should be expressed in the chloroplast along with the *ectA* gene in order to investigate whether any ectoine is synthesized leading to increased salt tolerance.

## 2 AIMS OF STUDY

### *The main goal of the project:*

- To express *ectB* gene in the chloroplast of *Chlamydomonas reinhardtii*, along with the *ectA* gene.
- To investigate whether the salt tolerance in *Chlamydomonas* cells transformed with the *ectA/B* genes is improved in comparison to wild type cells.

### *The main goals were further divided into sub-goals*

- Clone the *ectB* gene along with its gene cassette into a chloroplast transformation vector that already harbours an *ectA* gene.
- Introduce the transformation vector containing the *ectB* gene into the chloroplast of *Chlamydomonas reinhardtii* by microprojectile bombardment.
- Detect the *ectB* gene and mRNA in transformants to measure expression.
- Determine salt tolerance of selected transformants.
- Identify and analyze accumulated ectoine in cell extracts by High-performance liquid chromatography (HPLC).

## 3 METHODS AND MATERIALS

### 3.1 *Escherichia coli* methods

Competent *E. coli* TB1 cells were used in cloning.

#### 3.1.1 Liquid medium for growth with kanamycin (or ampicillin)

Antibiotics are degraded by high temperatures, and are therefore added to the LB medium at approximately 50 °C after autoclaving.

Procedure:

- Prepare 1 L Luria-Bertania (LB) medium by mixing:  
10 g NaCl, 5 g yeast, 10 g tryptophan, 200 µL 5M NaOH in 1 L dH<sub>2</sub>O.
- Autoclave the LB medium for approx. 20 minutes.
- Let the solution cool and supplement with 1mL kanamycin [50 µg/mL] or 1mL ampicillin [60 µg/mL].
- The solution is stored in Erlenmeyer flasks at room temperature.

#### 3.1.2 Solid medium for growth with kanamycin (or ampicillin)

- Prepare 1L Luria-Bertania (LB) medium by mixing:  
10 g NaCl, 5 g yeast, 10 g tryptophan, 200µL 5M NaOH in 1 L distilled water.
- Add 15 g 1.5% agar the LB medium and autoclave for about 20 minutes.
- Let the solution cool and supplement with 1mL kanamycin [50 µg/mL] or 1mL ampicillin [60 µg/mL].
- The solution is poured into sterile plastic plates under a sterile hood, and allowed to solidify.
- After solidification, the plates are inverted and stored 4 °C.

#### 3.1.3 Preparation of competent *E. coli* TB1 cells (CaCl<sub>2</sub> method)

Use sterilized equipment during this procedure:

- Grow TB1 *E. coli* cells in 3 mL LB medium at 37 °C overnight on a rotating wheel.
- Inoculate 100 mL LB medium with 2 mL of the culture in a 250 mL Erlenmeyer flask. Grow at 37 °C on a shaker.
- The absorbance of the culture might be checked at 600nm in a 1 cm plastic cuvette (with LB medium as standard).
- After approx. 2.5 hours, when OD<sub>600nm</sub> is around 0.6, harvest the cells by centrifugation [5000rpm for 10 min at 4 °C] in two 50 mL capped plastic tubes.
- Discard supernatant and resuspend the pellet in each tube in 20 mL ice-cold sterile 0.1M CaCl<sub>2</sub>. Leave tubes on ice for 10 minutes.

- Repeat the centrifugation as previously and discard supernatant. Resuspend each pellet in ice-cold sterile 2 mL 0.1M CaCl<sub>2</sub> with 15% glycerol.
- Transfer 50µL aliquots to each 1.5 mL microfuge tubes on ice, and freeze cells immediately in freezer [-80 °C] or in liquid nitrogen. Cells remain competent for several weeks. Competency may be checked by transformation with a control plasmid, like pUC or pBluescript, at a concentration of 1 ng/µL.

### **3.1.4 Transformation of competent *E. coli***

- Melt an aliquot [50 µL] of frozen competent cells on ice, and add 3 µL of ligation mix or pure DNA [1 ng/µL] or 3 µL of a ligation reaction. Mix and leave tube on ice for 30 minutes.
- Heat shock the cells in a water bath for 90 seconds at 42 °C. Cool down immediately on ice.
- Transfer the cells to a Falcon tube with loose cap and add 0.8 mL sterile LB medium.
- Incubate the mixture at 37 °C on a rotating wheel for 45 - 60 minutes.
- Plate the cell suspension as described in section 3.1.5.

### **3.1.5 Growth of *E. coli* on agar plates**

The following procedure should be done in a sterile hood.

- Sterilize the glass rod spreader in ethanol [96%] and then pass through a burning flame. Cool it down in air.
- Pipette 75 µL *E. coli* culture (cell suspension) onto antibiotic containing agar plate.
- Spread the cells on the plate with the glass rod.
- Allow excess liquid to dry into agar.
- Invert the plate and incubate at 37 °C for 16 - 20 hours.
- Store plate at 4 °C.

### **3.1.6 Small scale growth of *E. coli***

- Add 3 mL sterile LB medium containing either ampicillin [60 µg/mL] or kanamycin [50 µg/mL] to a Falcon tube with loose cap.
- Inoculate the medium with cells from a single colony from the growth plate or add 5 µl of a liquid culture.
- Incubate the cells overnight on a rotating wheel at 37 °C.
- The tube can be used for plasmid miniprep isolation (section 3.1.8) the next day.

### **3.1.7 Storage of *E. coli* at -80 °C**

- For long term storage of cells, mix 0.7 mL of the *E. coli* culture with 0.3 mL of 50% glycerol in a 2 mL tube with screw cap.



- Store in freezer at -80 °C.

### 3.1.8 Miniprep plasmid isolation from *E. coli*

- Transfer 1.5 mL of an *E. coli* culture into a 1.5 mL microfuge tube.
- Centrifuge at 13,000 g for about 30 seconds.
- Discard supernatant, and resuspend the bacterial pellet in 100 µL ice-cold TEG buffer.
- Leave for 5 minutes at room temperature.
- Add 200 µL of NaOH/SDS [0.2 N, 1% (w/w)] solution prepared fresh. Mix by inversion, and incubate on ice for 5 minutes.
- Add 150 µL ice-cold potassium acetate [5M potassium, 3M acetate]. Mix by inversion, and incubate on ice for 5 minutes.
- Centrifuge at 13,000 g at 4 °C for 5 minutes.
- Transfer supernatant to a new microfuge tube, and add 410 µL of phenol/chloroform/isoamyl alcohol [25:24:1]. Mix by vortexing.
- Centrifuge at 13,000 g for 2 minutes.
- Transfer upper phase to a new tube and add 410 µL of chloroform/isoamyl alcohol [24:1]. Mix by vortexing.
- Centrifuge at 13,000 g for 2 minutes.
- Transfer 310 µL of upper phase to a new tube. Add 750 µL of ice-cold ethanol [96%]. Mix by vortexing, and leave on ice for 10 minutes.
- Centrifuge at 13,000 g for 10 minutes at 4 °C.
- Discard supernatant, and add 1mL of ethanol [70%] to the pellet. Mix by inversion.
- Centrifuge at 13,000 g for 5 minutes at 4 °C.
- Discard supernatant, and dry the pellet by leaving at room temperature for 10 minutes, or dry in a vacuum centrifuge.
- Dissolve the pellet in 15 µL of dH<sub>2</sub>O.

A yield of 1- 3µg DNA is expected when isolating plasmid from a 1.5 mL cell culture.

### 3.1.9 Maxiprep plasmid isolation from *E. coli*

- Inoculate 100 mL LB medium, supplemented with either ampicillin [60 µg/mL] or kanamycin [50 µg/mL], with 5 µL LB of an *E. coli* culture or a colony from a plate and grow the culture overnight at 37 °C on a shaker.
- Harvest the cells by centrifugation [6,000 rpm] at 4 °C for 5 minutes.
- Discard the supernatant. Resuspend the cells in 3.6 mL ice-cold TEG buffer and transfer them to a 50 mL plastic tube.
- Add 0.4 mL lysozyme [10 mg/mL] in freshly prepared TEG and mix. Leave at room temperature for 5 minutes and then on ice for 5 minutes.
- Add 8 mL NaOH/SDS [0.2N, 1% (w/w)] solution prepared freshly. Mix by inversion, and leave on ice for 5 minutes.

- Add 6 mL ice-cold potassium acetate [5M potassium, 3M acetate]. Mix by inversion and incubate on ice for 5 minutes.
- Centrifuge at 6,000 g for 10 minutes at 4 °C.
- Transfer supernatant to a new tube by pouring it through a cheesecloth-filter.
- Add 12.5 mL isopropanol. Mix by vortexing and incubate at room temperature for 15 minutes.
- Centrifuge at 9,000 g for 10 minutes at room temperature.
- Discard supernatant. Leave the tube in room temperature in an inverted position for drying for 10 minutes.
- Resuspend pellet in 3 mL TE [50mM Tris (pH 8), 1mM EDTA] buffer.
- Determine the weight of the solution and add TE buffer [50mM Tris (pH 8), 1mM EDTA] until the solution has a total weight of 4.2 grams.
- Add 4.5 g CsCl to the solution, mix, and allow solution to warm to room temperature.
- Add 0.5 mL ethidium bromide [10 mg/mL], mix by gently swirling the solution and centrifuge at 6,000 g for 5 minutes at room temperature.
- Transfer supernatant to a Beckman OptiSeal tube, and load tube into VTi 65.2 rotor.
- Centrifuge at 50,000 g at 15 °C for 15 hours or more. The rotor should decelerate without brake.
- Illuminate the tube with 350 nm UV-light and extract the middle band containing DNA with a 2 mL syringe. Expected volume is around 0.5 mL.
- Remove ethidium bromide by repeated extraction with 0.5 mL isopropanol/water [7:1] up to five times.
- Dialyze against sterile 200 mL TE buffer at 4 °C to remove CsCl. Replace with fresh buffer every hour, twice or three times.
- Transfer DNA solution to a tube with screw cap, and store in -20 °C freezer.
- Determine DNA concentration by measuring OD<sub>260nm</sub>, with a 1:100 dilution as in section 3.2.3.

A yield of about 150 – 400 µg DNA is expected.

## 3.2 DNA methods

### 3.2.1 Agarose gel electrophoresis

1% agarose gel was used throughout this project, prepared as follows:

- Dissolve 0.6g agarose in 60 mL TAE [1X] buffer by boiling.
- Cool the solution down to 50 °C, and add 10µL ethidium bromide [1 mg/mL].
- Pour the solution into a tray, insert the comb, and wait until agarose is solid.
- Mix 9 µL DNA sample with 1µL agarose gel loading buffer, and load 10µL into each well in the gel.
- Load a 1kb plus DNA ladder (Invitrogen) in another well for estimation of DNA size and concentration.

- Run the agarose gel in electrophoresis buffer at 90V for 30 minutes or longer.
- Visualize the bands under UV-light.

### 3.2.2 Purification of DNA fragments from an agarose gel

After the gel electrophoresis separate the DNA fragments according to size. Bands of interest can be sliced out and purified by an Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare).

Procedure:

- Cut out an agarose slice which contains the DNA fragment of interest, and put into a 1.5 mL Eppendorf tube.
- Calculate the weight of agarose gel slice by weighing.
- Add 10 mL Capture buffer (type 3) to each 10 mg agarose gel.
- Incubate at 60 °C until the gel is completely dissolved. Mix by inverting the tube occasionally.
- Transfer 800 µL of the Capture buffer sample to a GFX Microspin column assembled with a collection tube beneath. Incubate at room temperature for 1 minute.
- Spin the assembled column and collection tube at 16,000 g for 30 seconds. Discard the flow. Repeat this step if there is more sample left.
- To remove leftovers from agarose gel, add 500 µL Wash buffer type 1 to the GFX Microspin column and spin at 16,000 g for 30 seconds.
- Discard the collection tube and move the GFX Microspin column to a 1.5 mL Eppendorf tube.
- Add 10-50 µL Elution buffer type 4 or type 6 to the centre of the GFX microspin column and incubate at room temperature for 1 minute.
- Spin at 16,000 g for 1 minute to isolate the DNA from the membrane of the GFX Microspin column. The DNA can be stored at -20 °C.

### 3.2.3 DNA and RNA quantification

*Determination by UV light absorption*

- Calibrate the spectrophotometer at 260nm with a control sample (usually dH<sub>2</sub>O, as dH<sub>2</sub>O is used to resuspend DNA).
- Dilute the DNA sample if needed.
- Measure the absorption at 260nm. OD 1.0 = 50 µg DNA/mL [40 µg RNA/mL].

*Dot spot method*

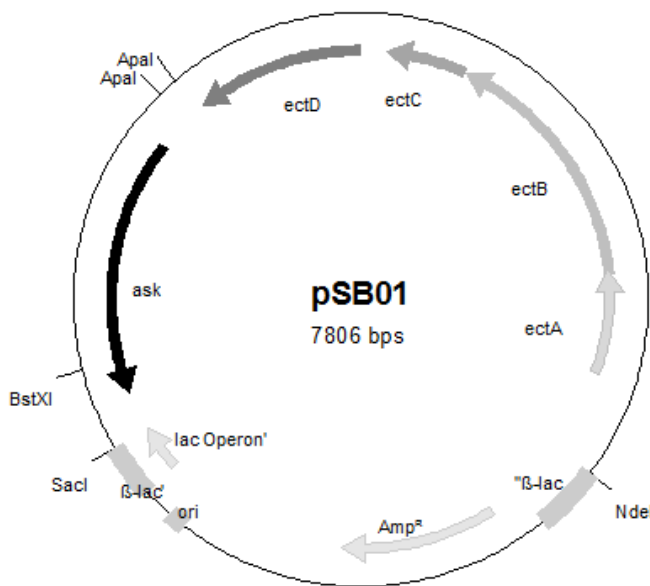
If the DNA sample contains impurities UV light absorption cannot be used. Another way to estimate the amount of DNA is by the Dot spot method, where the amount of DNA is proportional to the amount of fluorescence.

- Prepare DNA standards in water solutions with several concentrations [0 ng/μL, 2.5 ng/μL, 5 ng/μL, 7.5 ng/μL, 10 ng/μL, 15 ng/μL, and 20 ng/μL].
- On a UV-light transparent surface, add 2 μL ethidium bromide [2 μg/mL] to each 2 μL drop of DNA standards. Add 2 μL of the DNA sample(s), might be diluted to fit the range of standards. Mix well within the drop by pipetting up and down.
- Examine the intensity of fluorescence of the drops under UV-light and compare with the intensity of the DNA standards to roughly estimate the DNA concentration.

### 3.3 Subcloning

#### 3.3.1 Plasmids

The pSB01 vector carries the cloned *ectABCD* operon from *Pseudomonas stutzeri* (Figure 3.1), which is under the control of the *P. stutzeri* promoter region (Seip et al., 2011). The vector was provided by Dr. Erwin Galinski (University of Bonn, Germany).

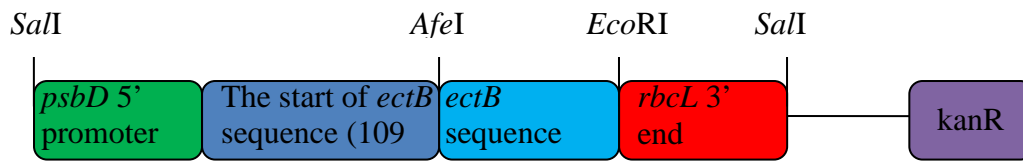


**Figure 3.1:** Vector map of pSB01 (Seip et al., 2011).

After isolating *ectB* and *ectC* genes from pSB01, the genes were subcloned into the pBluescript® II SK<sup>+</sup> phagemid (Stratagene).

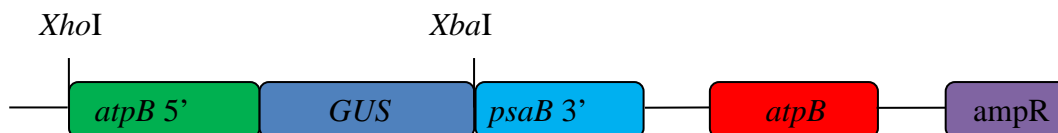
The *ectB* coding sequence was later cloned into the *psbD-rbcL\_pMK-RQ* plasmid. The *psbD-rbcL\_pMK-RQ* was synthesized by the GeneArt® service of Life Technologies™. The construct was designed to contain a *Chlamydomonas* chloroplast *psbD* promoter (Figure 3.2),

the beginning of *ectB* gene (about 109 bp), and the *rbcL* 3' end region. Moreover, an *EcoRI* and a *BsrDI* restriction site were added to facilitate cloning.



**Figure 3.2:** psbD-ectB-rbcL in psbD-rbcL\_pMK-RQ plasmid. The *AfeI* and *EcoRI* restriction sites were used for integrating the rest of the *ectB* coding sequence into psbD-rbcL\_pMK-RQ. *SalI* restriction sites at each end were used to release the *ectB* construct from the psbD-rbcL\_pMK-RQ plasmid. The plasmid confers kanamycin resistance.

The *ectA* transformation vector used (Figure 3.3) had the backbone of the pCrc32 plasmid (Blowers et al., 1993). The pCrc32 plasmid contains a portion of the chloroplast inverted repeat, a *GUS* (bacterial  $\beta$ -glucuronidase) gene, the chloroplast *atpB* gene and the 3' region of the *psaB* gene. By digesting the pCrc32 at the unique *XhoI* and *XbaI* sites, the *atpB* 5' end and *GUS* sequences were removed and replaced by the *rbcL* 5' promoter and the *ectA* gene (Lunde, 2012).



**Figure 3.3:** Map of plasmid pCrc32. The *ectA* gene along with the *rbcL* 5' promoter were cloned in between the *XhoI* and *XbaI* sites before cloning of *ectB* gene. The plasmid confers ampicillin resistance.

### 3.3.2 Polymerase chain reaction (PCR)

The polymerase chain reaction was performed with a Taq polymerase (Sigma-Aldrich) in a T1 Thermocycler (Biometra). Primers were synthesized by Eurofins MWG Operon (Germany) and designed to contain an *EcoRI* and a *BsrDI* restriction site. The sequences can be found in Table 3.1.

For a 100  $\mu$ L reaction cocktail:

- In a 1.5 mL Eppendorf tube add 65 $\mu$ L dH<sub>2</sub>O.
- Add 10  $\mu$ L 10x DyNzyme buffer.
- Add 3  $\mu$ L dNTPs [10mM]

- Add 10 µL plasmid template DNA [1 ng/µL]
- Add 5 µL of forward primer [10 pmol/µL] and 5 µL of reverse primer [10 pmol/µL].
- Add 2 µL of the thermostable DNA polymerase [2 u/µL].
- Mix with a pipette, centrifuge briefly to collect all liquid in bottom of the tube.
- Transfer 50 µL to each PCR tube and start the PCR thermal cycler.

PCR reaction:

Step 1: 98 °C, 3 minutes

Step 2: 98 °C, 15 seconds

Step 3: 56 °C, 1 min

Step 4: 72 °C, 45 seconds, repeat steps 2-4 30 times

Step 5: 72 °C, 10 minutes

Primer	Sequence	Restriction enzyme	Primer name
Forward	5'-CAC <u>GCAATG</u> AAACTTTTGAAGT-3'	<i>BsrDI</i>	5' ectB-BsrDI
Reverse	5'- ATTGCGAATTCCTCAGGAAGCTGGTTC -3'	<i>EcoR1</i>	3' EcoR1

**Table 3.1:** An overview of the primers used amplifying the *ectB* gene. The table shows the forward and reverse primer used to amplify the *ectB* gene for cloning into psbD-rbcL\_pMK-RQ. Restriction sites are underlined.

PCR products were checked on an agarose gel.

### 3.3.3 Restriction digestion of DNA

The restriction digestions were performed with restriction enzymes from New England Biolabs (NEB) according to the protocols from the manufacturer.

### 3.3.4 Partial restriction digestion of DNA

*The partial digestion was performed twice.*

20 µg of plasmid psbD-rbcL\_pMK-RQ was digested with 5 units of restriction enzyme *BsrDI*. The DNA is digested at 65 °C for 7 minutes in a volume of 50 µL. In order to stop the reaction immediately, cool it down on ice and add gel loading buffer before running on an agarose gel.

20 µg of Bluescript II SK<sup>+</sup> plasmid containing the *ectB* gene, was digested with 5 units of *BsrDI*. The DNA was digested at 65 °C for 7 minutes in a volume of 50 µL.

### 3.3.5 Dephosphorylation

In order to prevent self-ligation, the 5' phosphates have to be removed prior to ligation.

- Dissolve 5µg DNA fragment in 100µL appropriate buffer (0.5µg in 10µL).
- 1µL calf intestine phosphatase (CIP from NEB) and dH<sub>2</sub>O to a final volume of 100µL. Mix and incubate at 37 °C for 1 hour.
- To inactivate phosphatase, add 20µL EGTA [100mM] and incubate for 10 minutes at 65 °C.
- Do an extraction of sample with 120µL phenol/chloroform/isoamyl alcohol [25:24:1] and then with 120µL chloroform/isoamyl alcohol [24:1].
- Collect the supernatant from the water phase (upper phase).
- Precipitate the DNA by adding 1/10 of the volume of Na-acetate [3M] and 2 volumes of ethanol [96%]. In order for the precipitation to occur, leave the tube in freezer for up to 1 hour [-20 °C].
- Harvest the DNA by centrifugation at 4 °C for 10 minutes. Wash pellet with ethanol [70%] and dry.
- Resuspend the DNA in appropriate volume of either dH<sub>2</sub>O or TE buffer. DNA concentration may be determined by Dot spot method, as described in section 3.2.3.

### 3.3.6 Ligation

Ligations were performed with T4 DNA ligase (NEB), in a total volume of 10 µL. Usually the insert:vector ratio was 1.3:1 (for inserts of 0.5-3 kb).

Procedure:

- Add DNA-insert, DNA-vector and dH<sub>2</sub>O to a volume of 6.5µL.
- Incubate 5 minutes at 45 °C.
- Cool to room temperature and add 1µL T4 DNA ligase buffer [10x].
- Add 2µL polyethylene glycol (PEG) 8000 [30%, w/v].
- Add 0.5µL T4 DNA ligase enzyme [3 u/µL].
- Incubate for 3 hours at 19 °C.
- Leave the ligation mixture in room temperature for 30 minutes before using it in a transformation.

## 3.4 *Chlamydomonas reinhardtii* methods

The wild type strain (CC-124) of *Chlamydomonas reinhardtii* was provided by the Chlamydomonas Genetics Centre at Duke University in North Carolina, USA. The *Chlamydomonas* mutant strain (CC-373) was also obtained from Duke University. The non-photosynthetic mutant was kept in dark (or dim light) in high salt high acetate (HSHA) media.

### 3.4.1 Liquid media for growth of *Chlamydomonas*

HS (high salt)

For 1 litre:

20 mL salt stock (see below)

20 mL phosphate stock (see below)

1 mL trace elements (Hutner)

HSHA (high salt high acetate)

For 1 litre:

20 mL salt stock (see below)

20 mL phosphate stock (see below)

1 mL trace elements (Hutner)

2.5 g potassium acetate

Salt stock (50x):

For 500 mL

12.50 g  $\text{NH}_4\text{Cl}$

0.50 g  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$

0.25 g  $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$

Phosphate stock (50x):

For 500 mL

47 g  $\text{K}_2\text{HPO}_4 \cdot 3 \text{H}_2\text{O}$

18 g  $\text{KH}_2\text{PO}_4$

### 3.4.2 Solid media for growth of *Chlamydomonas*

Procedure for 1 L media:

- Prepare 1 L high salt high acetate or high salt (HSHA/HS) media with 1.5 % agar (15g agar/L). Autoclave for 20 minutes.
- Cool down solution to 50 °C.
- Pour into sterile plates and let solidify.
- Wait until solidification, before turning the plates upside down in order to avoid water condensation. Seal in plastic bags and store at room temperature.

### 3.4.3 Culturing the photosynthetic mutant of *Chlamydomonas* for transformation

The cells are light-sensitive and should at all times be kept in dark or dim light.

Inoculate 100 mL of HSHA medium with cells of the non-photosynthetic *Chlamydomonas* mutant and grow for 2 days on a shaker.

- Transfer about 5 mL of the culture to a new flask with 100 mL HSHA media.
- Grow the culture on a shaker for 2 days.
- Transfer culture to a flask with 500 mL HSHA medium and grow on shaker for 1 day.



### 3.4.4 Plating the photosynthetic mutant of *Chlamydomonas* for transformation

The cells were plated on HSHA agar right before the transformation. All equipment and solutions should be sterile.

- Centrifuge 500 mL of the culture at 4000 rpm for 5 minutes at room temperature in two sterile centrifuge tubes.
- Discard the supernatant and resuspend cells in 500  $\mu$ L HSHA media.
- Melt agar medium [0.11% agar in HSHA] in a microwave oven. Add 900  $\mu$ L to two preheated [42 °C] sterile microfuge tubes.
- Let the tubes cool down to 42 °C.
- Add 100  $\mu$ L of the resuspended *Chlamydomonas* cells to each microfuge tube, and mix gently.
- Transfer 300  $\mu$ L of the cell suspension to each HSHA agar plate [5 cm in diameter]. Spread the liquid evenly on the plates by shaking the plate.
- Store the plates in the dark for about 3 hours before microprojectile bombardment.

### 3.4.5 Microprojectile bombardment

The transformation of foreign gene into the *Chlamydomonas* chloroplast was performed with the particle delivery system (PSD1000He, Bio-Rad), with helium gas at a pressure of 1350 psi.

Procedure:

- Precipitate the DNA sample (transformation vector) to 0.6  $\mu$ m gold particles (Bio-Rad), according to the protocol provided by manufacturer of the PDS. Resuspend carefully as DNA coated particles tend to aggregate.
- Before shooting, the inside of the particle delivery system (PSD1000He, Bio-Rad) should be sterilized with alcohol.
- Following the protocol for shooting and place the agar plates in dark at room temperature overnight.

### 3.4.6 Selection of the transgenic clones of *Chlamydomonas*

Transformants are selected by transferring the algae to HS agar plates and growing them photosynthetically in continuous light. Non-transformed cells will eventually die. The transfer should be done under a sterile hood. Wait 1-2 days until the liquid is dried before turning the plates upside down.

Procedure:

- Add 300  $\mu$ L high salt (HS) media evenly to each plate.
- Scrap all cells from the agar plate surface with a sterile metal rod.

- Transfer the media (now containing the cells) to a fresh HS agar plate with a pipette, and spread it on the agar plate with a glass rod.
- Place the HS agar plates under light. Wait 1-2 days before sealing the plates with parafilm so the liquid does not evaporate.
- Colonies should appear after approximately 2-3 weeks. These can be picked and transferred to an HS agar plate with a visual grid, and subsequently used to inoculate liquid cultures of 200 mL.

### 3.4.7 Photosynthetic growth of *Chlamydomonas* with 2% CO<sub>2</sub>

- Add approximately 100 mL of high salt (HS) medium to sterilized 250 mL glass tubes, and then add about 60 mL of a liquid *Chlamydomonas* culture.
- Place the glass tubes on a rack in a water bath at 32 °C and a light source.
- Connect the tubes to a source of 2% CO<sub>2</sub> in air. Make sure that there is equal amount of bubbling in each glass tube.
- After 2-3 days the cultures should be dark green and ready for harvesting.

### 3.4.8 Isolation of genomic DNA from *Chlamydomonas*

Use a culture of  $\approx 2 \times 10^6$  cells/mL.

- Centrifuge 40 mL culture at 5000 g for 5 minutes at 4 °C.
- Resuspend pellet in 0.75 cold DNA extraction buffer [100 mM Tris (pH 8.0), 50 mM Na<sub>2</sub>-EDTA, 0.5 M NaCl, 10 mM  $\beta$ -mercaptoethanol] and transfer the suspension to a 2 mL microfuge tube.
- Add 60  $\mu$ L SDS [21% w/v], mix, and incubate for 15 min at 65 °C.
- Cool down to room temperature and add 0.9 mL phenol [equilibrated with 0.1 M Tris, pH 8.0]. Mix carefully by inversion.
- Centrifuge at 13,000 g for 5 minutes at room temperature.
- Transfer 750  $\mu$ L of upper phase to a new 2 mL microfuge tube, add 750  $\mu$ L phenol/chloroform/isoamylalcohol [25:24:1], and mix by inversion.
- Centrifuge at 13,000 g at room temperature for 5 minutes.
- Transfer 650  $\mu$ L of upper phase to a new 1.5 mL microfuge tube, and add 650  $\mu$ L of isopropanol. Mix by inversion and incubate at room temperature for 5 minutes (or until precipitation occurs).
- Centrifugation at 4,000 g for 2 minute at room temperature. Discard supernatant and add 1mL of ice-cold ethanol [70%]. Mix by inversion.
- Centrifuge at 13,000 g at room temperature for 2 minutes. Discard supernatant and dry pellet in a vacuum centrifuge, or by incubation at room temperature.
- Centrifuge at 13,000 g at room temperature for 2 min. Discard supernatant and dry pellet in a vacuum centrifuge, or by incubation at room temperature.

- Resuspend pellet in 90  $\mu\text{L}$  TE buffer [10 mM Tris (pH 8.9), 1 mM  $\text{Na}_2\text{EDTA}$ ], add 10  $\mu\text{L}$  RNase A [1 mg/mL], and incubate at 37 °C for 1 hour.
- Extract the mixture once with 100  $\mu\text{L}$  phenol/chloroform/isoamylalcohol [25:24:1] and once with 100  $\mu\text{L}$  chloroform/isoamylalcohol [24:1].
- Precipitate DNA in freezer with 0.3 M Na-acetate and 2 volumes ethanol [96 %], for up to 1 hour or overnight.
- Centrifuge at 13,000 g at 4 °C for 10 minutes. Discard supernatant, and add 1 mL of ice-cold ethanol [70 %]. Mix by inversion.
- Centrifuge at 13,000 g at 4 °C for 5 minutes. Discard supernatant and dry pellet in a vacuum centrifuge, or by incubation at room temperature.
- Resuspend DNA in 20  $\mu\text{L}$   $\text{dH}_2\text{O}$ .
- Measure DNA concentration (chapter 3.2.3). Expected yield is about 4  $\mu\text{g}$ .

### 3.4.9 RNA isolation from *Chlamydomonas*

All work was done with RNase-free material and solutions. The cells were grown in 12-hour light/ dark cycles to a concentration of about  $2 \times 10^6$  cells/mL. Always keep samples on ice.

Procedure:

- Centrifuge 40 mL *Chlamydomonas* culture at 5,000 g for 5 minutes at 4 °C.
- Discard the supernatant and resuspend the pellet in 1.5 mL ice-cold lysis buffer [0.6M NaCl, 200mM tris (pH 8.0), 10mM  $\text{Na}_2\text{EDTA}$ ].
- Add 150  $\mu\text{L}$  RNase inhibitor [200mM vanadyl ribonucleoside (NEB)].
- Transfer cells to a 15 mL tube containing 2 mL phenol [equilibrated with 0.1 M tris pH 8.0] and 1.5 mL SDS [4 %] preheated to 65 °C. Mix.
- Incubate for 15 minutes at 65 °C, mix occasionally by shaking.
- Cool down on ice and add 1 mL ice-cold chloroform/isoamylalcohol [24:1]. Mix.
- Centrifuge at 8,000 g for 5 minutes at 4 °C.
- Transfer 3 mL of the upper phase to a new 15 mL tube containing 3 mL ice-cold phenol/chloroform/isoamylalcohol [25:24:1]. Mix.
- Centrifuge at 8,000 g for 5 minutes at 4 °C.
- Transfer 2.5 mL of the upper phase to another 15 mL tube containing 2.5 mL ice-cold phenol/chloroform/isoamylalcohol [25:24:1]. Mix.
- Centrifuge at 8,000 g at 4 °C for 5 minutes.
- Transfer 2 mL of the upper phase to a new 15 mL tube containing 2.5 mL ice-cold isopropanol and 250  $\mu\text{L}$  Na-acetate [3M, pH 5.2]. Mix and incubate at -20 °C for at least 1 hour.
- Centrifuge at 12,000 g for 15 minutes at 4 °C. Discard supernatant, and leave the tube inverted on a paper towel for 10 minutes in order to completely dry the pellet.
- Resuspend pellet in 300  $\mu\text{L}$  DEPC-treated  $\text{H}_2\text{O}$ , transfer to a 1.5 mL microfuge tube and add 100  $\mu\text{L}$  ice-cold LiCl [3M]. Incubate on ice for 2 hours.
- Centrifuge at 13,000 g for 30 minutes at 4 °C.
- Discard supernatant and resuspend pellet in 100  $\mu\text{L}$  DEPC-treated  $\text{H}_2\text{O}$ .

- Determine the concentration by diluting 10  $\mu$ L of the RNA solution in 1 mL DEPC-treated H<sub>2</sub>O, and measure OD<sub>260nm</sub> [OD 1.0 = 40  $\mu$ g RNA/mL].
- Precipitate the rest of the RNA solution and add 10  $\mu$ L Na-acetate [3M (pH 5.2)] and 200  $\mu$ L ice-cold ethanol [96%]. Incubate at -20 °C for 1 hour.
- Centrifuge at 13,000 g for 10 minutes at 4 °C. Discard supernatant and add 1 mL of ethanol [70%]. Mix by inversion.
- Centrifuge at 13,000 g for 10 minutes at 4 °C. Discard supernatant and dry the pellet in a vacuum centrifuge.
- Resuspend RNA in DEPC-treated H<sub>2</sub>O to a final concentration of 2  $\mu$ g/ $\mu$ L.

## 3.5 Analytical methods

### 3.5.1 Sequencing

Sequencing of the constructs was done by Eurofins MWG/Operon (Germany).

### 3.5.2 Preparation of radioactive probes for DNA and RNA blots

*ectA* and *ectB* probes used for hybridization in Southern and Northern analyses were full-length coding sequences isolated from plasmids by restriction cutting. The probes were labelled as follows:

- Add 10.5  $\mu$ L dH<sub>2</sub>O and 2  $\mu$ L template DNA [100-200 ng/ $\mu$ L] in a 1.5 mL Eppendorf tube.
- Denature the mixture in boiling water bath for at least 5 minutes.
- Add 5  $\mu$ L cold labeling buffer, 2.5  $\mu$ L BSA [4 mg/mL], 1.5  $\mu$ L dNTP mixture (deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyguanosine triphosphate [1mM of each, mixed 1:1:1])
- Add 2.5  $\mu$ L [25 microcurie ( $\mu$ Ci)] [ $\alpha$ -<sup>32</sup>P]-dCTP, and mix with a pipette.
- Add 1  $\mu$ L DNA polymerase I, (Klenow fragment) [2 units/mL] (NEB).
- Spin briefly and incubate at room temperature for 3 hours.
- Store at -20 °C. For hybridization use 1/2000 of the volume of the hybridization solution.

### 3.5.3 DNA Slot blot

- Add 3  $\mu$ L NaOH [5N] to a 1.5  $\mu$ L microfuge tube.
- Add 500 ng DNA dissolved in H<sub>2</sub>O [1-19  $\mu$ L], and raise total volume to 50  $\mu$ L by adding dH<sub>2</sub>O.
- Denature by incubating at 65 °C for 45 - 60 minutes. Cool down to room temperature.

- Add 50  $\mu$ L saline-sodium citrate buffer (SSC) [20x].
- Assemble the dot blot apparatus (PR 600 SlotBlot, Hoefer Scientific Instruments) according to instructions from manufacturer.
- In order to avoid air bubbles in sample slot, soak blotting membrane (nylon membrane from ZetaProbe; BioRad) in dH<sub>2</sub>O for 5 minutes, and then in SSC [10x] for 5 minutes.
- Place blotting membrane on dot blot apparatus, and pull 200  $\mu$ L SSC [10x] through sample slot by suction with a water-based vacuum pump.
- Turn off suction. Add 100  $\mu$ L SSC [10x] and denatured DNA sample in sample slot. Mix with a pipette in the slot. Turn on suction and wait until all liquid has been sucked through.
- Turn off suction, and wash slot by sucking through 200  $\mu$ L SSC [10x] till it is almost dry.
- Take out the blotting membrane, without touching the DNA binding area, and wrap it into plastic wrap.
- Crosslink DNA to membrane with 1200 energy in a CL-1000 Ultraviolet Crosslinker, UVP. Have the DNA binding side face the light.
- Hybridize the membrane with a radioactive probe and develop this by autoradiography (section 3.5.4 & 3.5.5)

### **3.5.4 Hybridization of DNA and RNA with radiolabelled probes**

This protocol is used for hybridization of radioactive probes using a random primer labelled probe of 1 to 3 kb size. All buffers should be preheated at 65 °C before use.

Procedure:

- Wash the blotting membrane with crosslinked DNA in dH<sub>2</sub>O.
- Place the blotting membrane with its backside against the wall in a hybridization tube. Add 2-3 mL of pre-hybridization buffer, and incubate at 65 °C on a rotating device for 15 minutes.
- Discard the hybridization buffer and hybridize at 65 °C with about 0.1 mL of pre-hybridization buffer per square cm of blotting membrane.
- Add random primer labelled probe until the volume corresponds to 1:2000 of the hybridization buffer.
- Incubate for about 24 hours on the rotation device.
- Discard the radioactive buffer, and wash membrane twice in buffer 1 for 5 minutes at 65 °C.
- Wash seven times, each for 5 min in buffer 2, and a final wash for 20 minutes in buffer 2.
- Wash the blotting membrane with dH<sub>2</sub>O, and wrap membrane in plastic foil. Verify radioactivity with a Geiger counter and visualize the hybridization by autoradiography.

### 3.5.5 Autoradiography

This procedure was performed with Kodak®BioMax®MS x-ray film and solutions.

- Adjust the blotting membrane by taping it onto an autoradiography cassette, and place an x-ray film and intensifying screen over it.
- Expose the film to -80 °C by using an intensifying screen overnight. Exposure to intensifying screen may last for longer, depending upon results from hybridization acquired by Geiger counter.
- Develop the film by dipping it in fixation solution for 1 min, then wash in H<sub>2</sub>O and finally in developer solution for 1 min.
- Wash away residues from previous solutions by H<sub>2</sub>O and dry the film in air or by hairdryer.

### 3.5.6 Southern analysis

- Isolate genomic DNA.
- Digest 1.5 µg genomic DNA with 30 units enzyme in a total volume of 20 µL. Digest for 4 hours.
- Precipitate the DNA with 2.2 µL Sodium acetate [3M (pH 5.2)], 67 µL ethanol [96%] and put in freezer for 1 hour.
- Centrifuge at 13000 g for 10 minutes at 4 °C to collect the precipitate, and wash the pellet with 1 mL ethanol [70%].
- Centrifuge at 13000 g for 5 minutes at 4 °C. Discard the supernatant. Dry pellet in vacuum centrifuge.
- Resuspend DNA in 10 µL TAE-buffer [1x] and add and 2 µL gel loading buffer.
- Run an agarose gel [1% agarose gel, at 90V].
- Visualize the gel under UV-light and take a picture.
- Incubate the gel in denaturation solution for 30 minutes.
- Incubate the gel in neutralization solution for 30 minutes.
- Add SSC [10X] to a capillary transfer apparatus.
- In the transfer apparatus, stack in the following order; three 3mm thick Whatman papers, a nylon membrane (BioRad), the gel, 2 Whatman papers and some paper towels on top. Remember to place the gel in the right orientation and avoid bubbles.
- Wash the nylon membrane in SSC [2x].
- Crosslink the DNA to the membrane with CL-1000 Ultraviolet Crosslinker, UVP (USA), at 1500.
- Hybridize the membrane with a probe and visualize the result by autoradiography.

### 3.5.7 Northern analysis

All equipment used in this procedure has to be RNAase-free.

Procedure:

- The electrophoresis chamber, comb and gel tray are sterilized overnight in H<sub>2</sub>O<sub>2</sub> [3%].
- Prepare a 1.3% agarose gel by dissolving 0.78g agarose in 37 mL DEPC-treated water, and bring it up to boil in a microwave.
- Cool down in a water bath to 65 °C.
- Add 12 mL MOPS [5x] and 11 mL formaldehyde [37%].
- Mix gently by swirling and pour into a gel tray. Place the comb in.
- Prepare the sample by mixing following solutions in given order:
  - 2.0 µL MOPS buffer [5x]
  - 3.5 µL formaldehyde
  - 3.5 µL ethidium bromide [100 µg/mL]
  - 10.0 µL formamide
  - 4.5 µL RNA [2 µg/µL]
- Incubate the samples at 65 °C for 15 minutes.
- Put the gel into the electrophoresis chamber and add running buffer to immerse the gel (315 mL DEPC-H<sub>2</sub>O, 90 mL MOPS [5x], and 45 mL formaldehyde).
- Pre-run the gel for 5 minutes at 60 mA.
- Cool down sample, centrifuge at max speed for 1 minute, and add 2 µL RNA gel loading buffer.
- Mix with a pipette and add 20 µL samples in each well.
- In order to get better resolution, run the samples at 60 mA for 10 minutes in reverse direction. And then in normal direction until the bromphenol blue band is at the bottom of the gel.
- Photograph the gel with a digital camera.
- Wash briefly in DEPC-treated H<sub>2</sub>O and transfer RNA to a nylon membrane following the protocol (BioRad Zetaprobe protocol).
- Transfer for 5.5 hours. Put membrane into SSC [2x]. Check gel under UV-light for complete transfer.
- Wrap membrane into plastic foil, and crosslink DNA to membrane with CL-1000 Ultraviolet Crosslinker, UVP, set to 1500 energy.
- Hybridize the membrane with a radioactive probe and develop by autoradiography.

### 3.5.8 Salt tolerance experiment

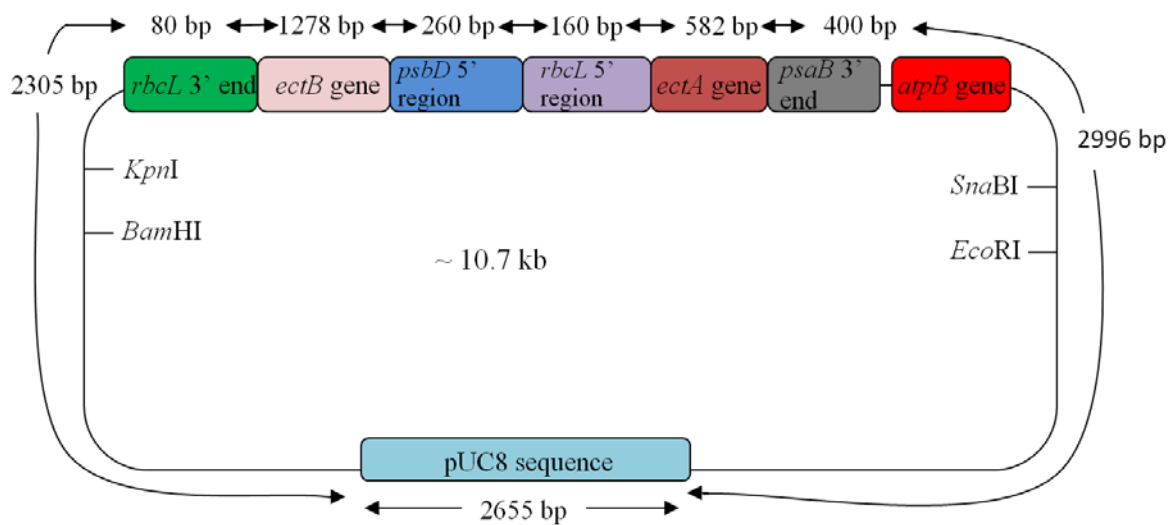
In order to check changes in salt tolerance, *Chlamydomonas reinhardtii* cells were grown phototrophically in HS media under bright light and 2% CO<sub>2</sub> in the presence of varying salt concentrations. In addition to wild type cells grown in either 0.25% or 0.5% NaCl, the mutant was grown at NaCl concentrations of 0.25%, 0.5% and 1.0%. Growth differences were evaluated visually.

## 4 RESULTS

### 4.1 The construct of ectA\_B transformation vector

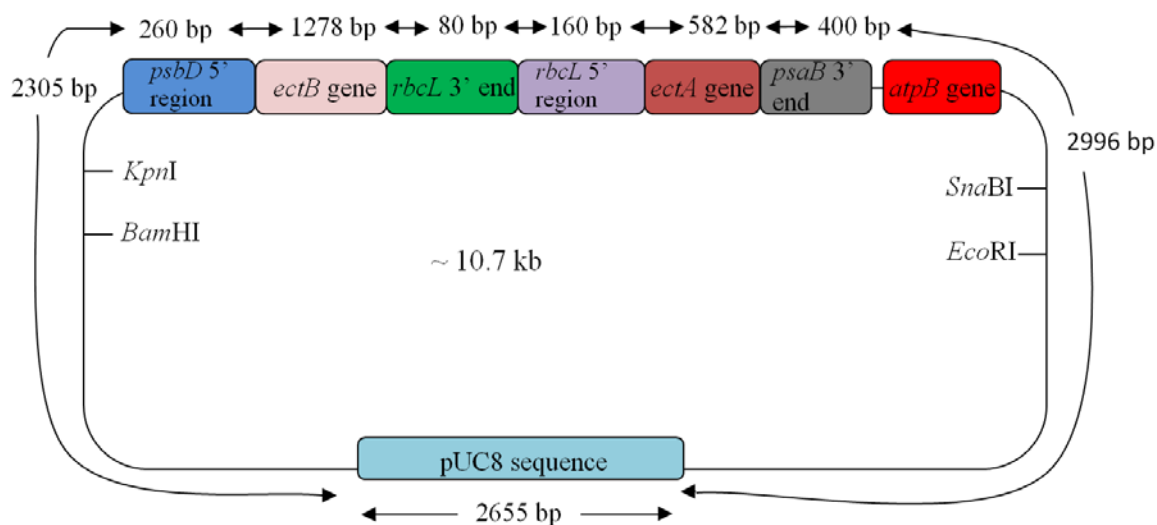
The first goal of the project was to construct a transformation vector that harbours the bacterial *ectA* and *ectB* genes for insertion into the chloroplast genome of *Chlamydomonas*. A transformation vector, already carrying the *ectA* gene and suitable for insertion of the *ectB* coding sequence, was available in the lab (Lunde, 2012). The *ectB* coding region, flanked by 5' and 3' regions of the *Chlamydomonas* chloroplast *psbD* and *rbcL* genes, respectively, was inserted in both orientations into an *XhoI* site located upstream of the *ectA* construct (Figures 3.2 and 3.3).

The different orientations of the *ectB* gene in the transformation vector are shown in Figures 4.1 and 4.2. In orientation 1 the *ectA* and *ectB* genes are transcribed divergently whereas in orientation 2 the two genes are transcribed in the same direction.



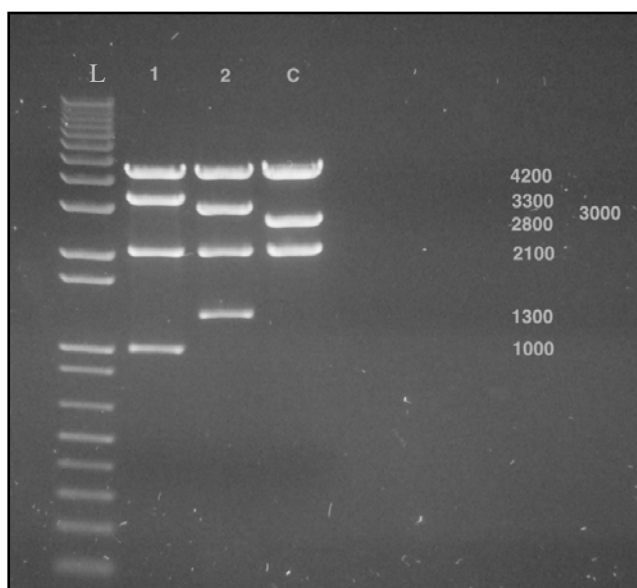
**Figure 4.1:** The *ectA\_B* transformation vector with *psbD* promoter, *ectB* gene and *rbcL* 3' end integrated in orientation 1. Sizes of the different vector elements are shown. The total size of the vector is about 10.7 kb. Not drawn to scale.





**Figure 4.2:** The *ectA\_B* transformation vector with *ectB* gene and its necessary elements integrated in orientation 2. See legend to Figure 4.1 for details.

The orientation of the *ectB* gene was verified by digesting the transformation vector with *Pst*I which cuts once in the *ectA* and once in the *ectB* gene (Figure 4.3). *Pst*I digestion releases a fragment of about 1000 bp when *ectB* is in the vector in orientation 1, whereas a fragment of about 1300 bp is released when *ectB* is in orientation 2. The two different orientations of *ectB* in the transformation vector were confirmed by sequencing (Appendix II and III).

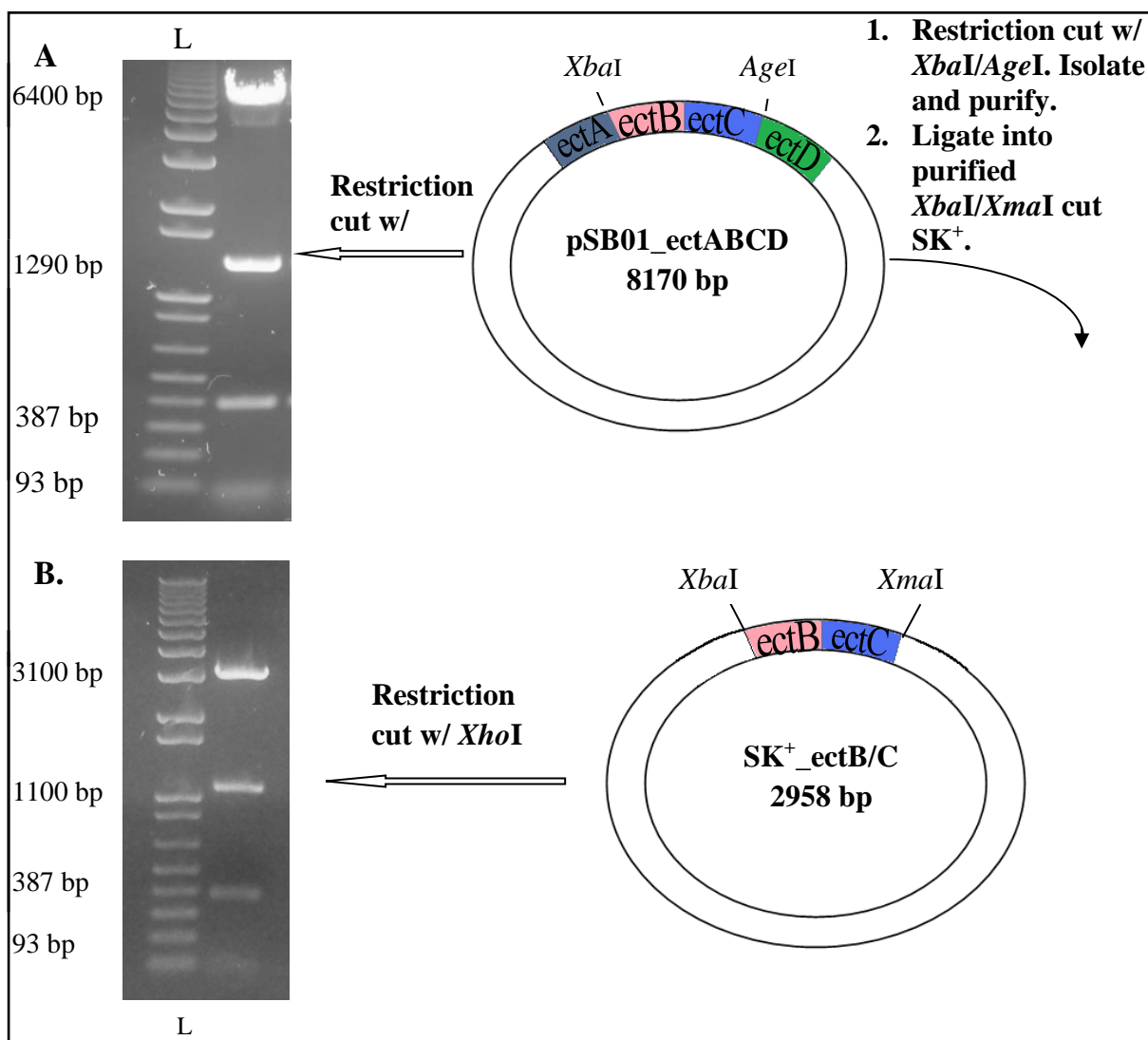


**Figure 4.3:** *Pst*I digestion of transformation vectors that carry the *ectB* gene in orientation 1 and 2. The samples were loaded as follows: L, 1 kb plus DNA ladder; 1, *ectB* construct in orientation 1; 2, *ectB* construct in orientation 2; C, control *ectA* vector without the *ectB* construct.

## 4.2 Subcloning of the *ectB* gene

Cloning of the *ectB* gene into the *Chlamydomonas* chloroplast transformation vector consisted basically of three steps: subcloning of the *ectB/C* genes from *P. stutzeri* into pBluescript II SK<sup>+</sup>, ligation of the *ectB* gene into the psbD-rbcL\_pMK-RQ plasmid and finally, the integration of the psbD-ectB-rbcL construct into the *ectA*-containing transformation vector. The *ectA\_B* transformation vector was shot into the chloroplast of mutant *C. reinhardtii* CC-373 cells.

The *ectB* coding sequence, which was obtained from Dr. Galinski (Seip *et al.*, 2011) was isolated from pSB01 plasmid containing the *Pseudomonas stutzeri* *ectABCD* operon. Prior to the cloning, it was verified that the *ectABCD* operon was present in the pSB01 plasmid by digesting with *Xho*I (Figure 4.4 A). *Xho*I digested the plasmid into four DNA bands (Figure 4.4 A) showing that the *ectABCD* genes were within the plasmid.

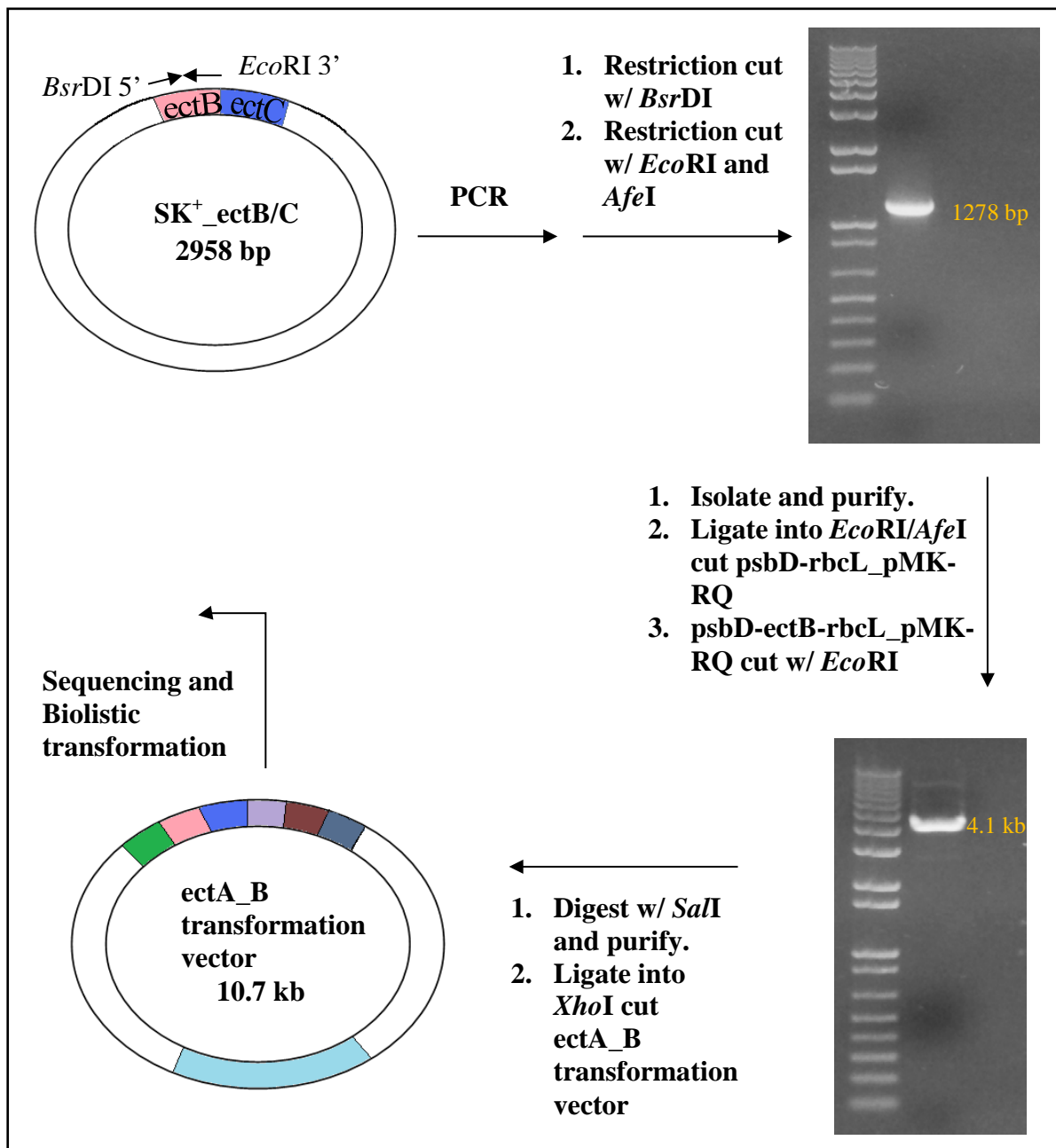


**Figure 4.4:** **A)** Plasmid pSB01 containing the *ectABCD* operon from *P. stutzeri* was digested with *XhoI* to check the presence of the *ectB* gene. *XhoI* cuts the *ectB* sequence in three positions releasing fragments of 93 bp and 387 bp. **B)** Digestion of *SK*<sup>+</sup>\_ectB/C to verify the presence of the *ectB* gene in pBluescript *SK*<sup>+</sup>. L, 1 kb plus DNA ladder.

The *ectB/C* coding sequences were subcloned into vector pBluescript *SK*<sup>+</sup> (Stratagene) by digesting vector pSB01 with *XbaI* and *AgeI* and inserting the *ectBC*-containing fragment ( $\approx$  1.7 kb) into the *XbaI* and *XmaI* sites of pBluescript *SK*<sup>+</sup> to create vector *SK*<sup>+</sup> *ectB/C* (*AgeI* and *XmaI* create compatible ends). The presence of *ectBC* in pBluescript *SK*<sup>+</sup> was verified by digesting *SK*<sup>+</sup>\_ectB/C with *XhoI* which releases 93 bp and 387 bp fragments from the *ectB* gene (Figure 4.4 B).

The *ectB* gene was amplified by PCR using *SK*<sup>+</sup>\_ectB/C as template and primers 5' *ectB* BsrDI and 3' *EcoRI* which introduced *BsrDI* and *EcoRI* sites, respectively, at the 5' and 3'

ends of the PCR product (Figure 4.5). The PCR product was purified on an agarose gel and digested with *EcoRI* and *AfeI*. The digested PCR product was cloned into the *AfeI* and *EcoRI* sites of vector psbD-rbcL\_pMK-RQ such that the *ectB* coding region became flanked by the 5' and 3' regions of the *Chlamydomonas* chloroplast *psbD* and *rbcL* genes, respectively. The resulting plasmid had a size of  $\approx 4.1$  kb.



**Figure 4.5:** PCR amplification of the *ectB* coding region for cloning into vector psbD-rbcL\_pMK-RQ, which is followed by cloning of the psbD-ectB-rbcL construct into the final *Chlamydomonas* chloroplast transformation vector. The 4.1 kb psbD-ectB-rbcL\_pMK-RQ plasmid (here digested with *EcoRI* to verify its size) was digested with *SalI* and the purified

psbD-ectB-rbcL fragment was ligated into the *Xho*I-digested ectA transformation vector. See Figure 4.1 for color codes of the segments. See Figure 3.2 for details of the final construct.

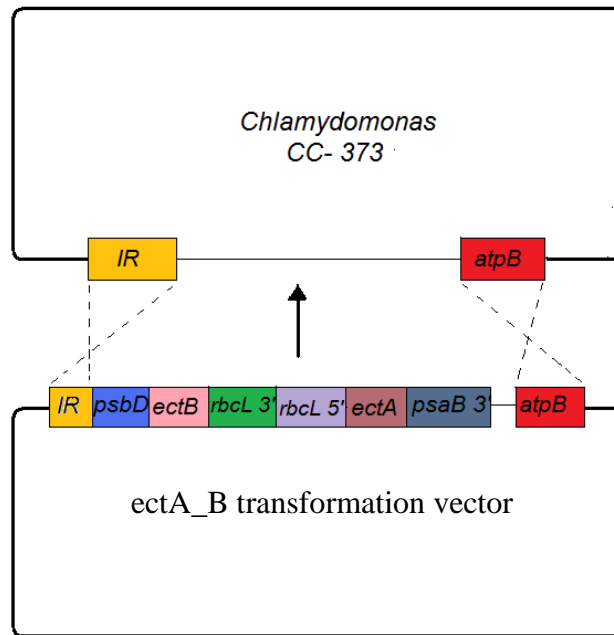
Next, the psbD-ectB-rbcL fragment was released from psbD-ectB-rbcL\_pMK-RQ by digestion with *Sal*I and cloned into the *Xho*I-digested, dephosphorylated ectA transformation vector (Figure 4.5).

#### **4.2.1 Complications during subcloning of the *ectB* gene**

The original strategy for subcloning was based on isolating the *ectB/C* sequence from the *P. stutzeri* vector and ligating it between the *Bsr*DI and *Eco*RI sites of plasmid psbD-rbcL\_pMK-RQ. It was later found that the backbone of the psbD-rbcL\_pMK-RQ vector had two *Bsr*DI restriction sites. Attempts to do the cloning by partial digestion of the vector were unsuccessful. The construct had, however, an *Afe*I site located between *Bsr*DI and *Eco*RI (Figure 3.2) that could be used for in-frame insertion of the *ectB* coding region.

#### **4.2.2 Microprojectile transformation and selection of phototrophic transformants**

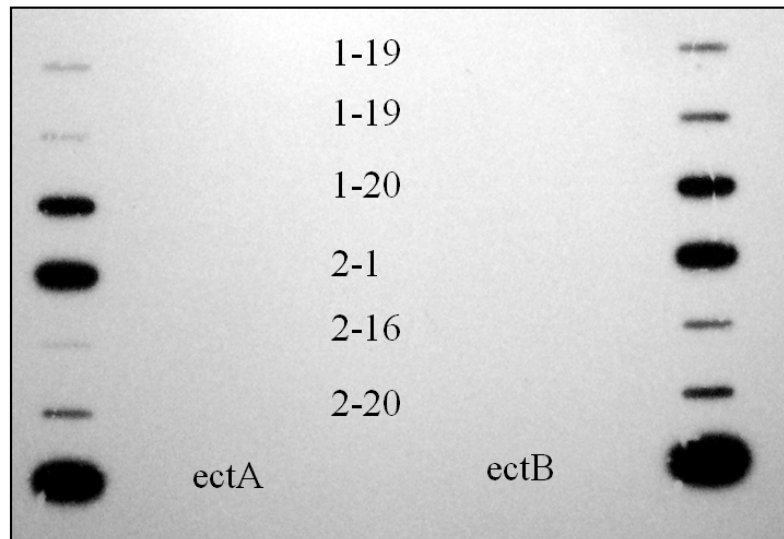
Gold particles were coated with the ectA\_B transformation vector and shot with high velocity onto agar-plated cells of the *Chlamydomonas* photosynthesis-deficient mutant CC-373. Homologous recombination between sequences in the chloroplast DNA and the plasmid inserted the *ectA/B* genes, and complemented a deleted segment of the *atpB* gene enabling phototrophic growth of the mutant (Figure 4.6). Afterwards the cells were transferred to HS medium agar plates for selection of positive photosynthetic transformants. Colonies seen after two to three weeks indicated that transformants with a restored *atpB* gene had been selected by exposure to bright light. Transformation through recombination events may have occurred in any of the 50 - 80 genome copies of the chloroplast.



**Figure 4.6:** Homologous recombination between the ectA\_B transformation vector and the corresponding segments in the chloroplast genome of the *atpB*-deficient mutant CC-373. Dashed lines indicate the recombination event between plasmid and genome sequences. This figure is showing a transformant with the *ectB* construct in orientation 2. IR; inverted repeat.

### 4.3 DNA slot blot analysis

In order to screen for *ectA/B*-positive transformants a DNA slot blot was performed. Genomic DNA isolated from six random transformant colonies with orientation 1 and 2 was denatured and covalently linked to a nylon membrane by exposure of UV-light. The membrane was hybridized to radioactively labelled *ectA* and *ectB* probes (chapter 3.5.2). Two of the six isolates, transformants termed 1-20 and 2-1, showed fairly strong autoradiogram signals (Figure 4.7). Thus, transformants 1-20 and 2-1 were chosen for further analysis.



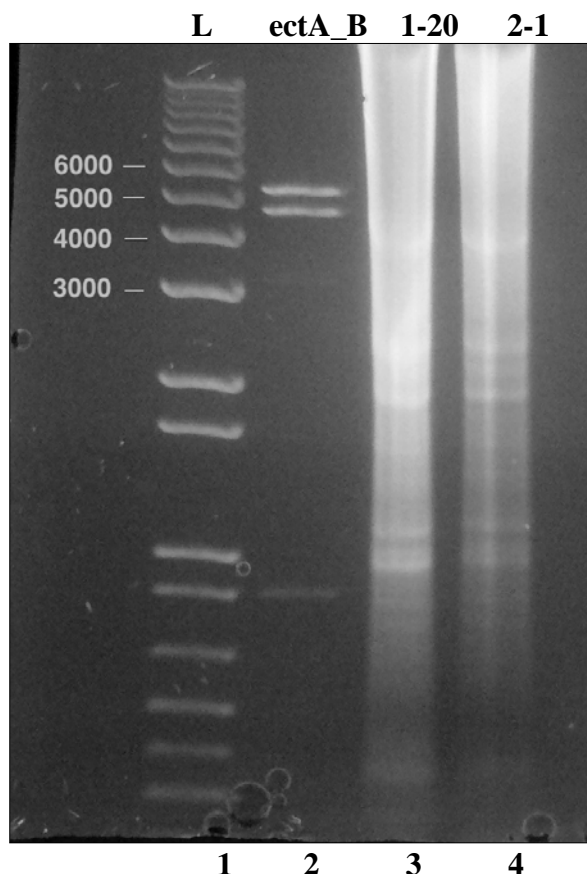
**Figure 4.7:** Autoradiogram of the DNA slot blot analysis with genomic DNA isolated from six *Chlamydomonas* chloroplast transformants. Samples from all six transformants with the *ectB* gene either in orientation 1 (1-19 and 1-20) or 2 (2-1, 2-16 and 2-20) were hybridized to radioactive *ectA* (left) and *ectB* (right) probes. Signal intensities are indicative of how many copies of the chloroplast genome harbour the *ectA/B* construct. Plasmids (1 ng) carrying the *ectA* and *ectB* genes were used as positive controls (bottom). The x-ray film was exposed at -70 °C for approximately 23 hours.

## 4.4 Analysis of transformants

### 4.4.1 Southern analysis

A Southern analysis was performed on the chosen transformants to determine the percentage of homoplasmicity. The homoplasmicity percentage indicates the number of chromosome copies in the *Chlamydomonas* chloroplast that carry the *ectA/B* sequence.

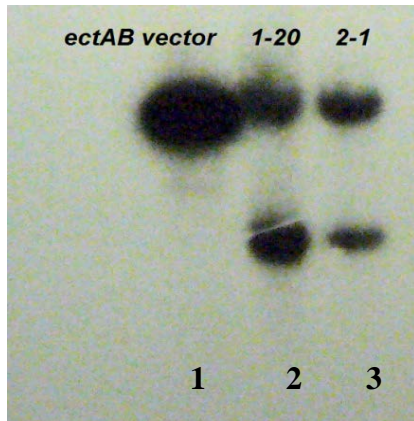
Total genomic DNA from transformants 1-20 and 2-1 was digested with *KpnI* and *SnaBI*. As shown in Figures 4.1 and 4.2, these restriction enzymes cut on either side of the inserted construct. The DNAs were loaded on an agarose gel and separated according to size (Figure 4.8). Genomic DNA appears as a smear in both wells due to many variously-sized fragments. Digestion of the *ectA\_B* transformation vector resulted in a couple of bands. Depending on integration of the *ectA/B* construct into the *Chlamydomonas* chloroplast genome, a fragment of ~ 5.2 kb containing the *ectA* and *ectB* construct or a fragment of ~ 2.5 kb can be expected after restriction cutting with *KpnI* and *SnaBI*.



**Figure 4.8:** Agarose gel electrophoresis of genomic DNA isolated from transformants 1-20 and 2-1, and of the *ectA/B* transformation vector digested with *KpnI* and *SnaBI*.



After transfer of the DNA fragments to a nylon membrane and hybridization to an *atpB* probe, the 5.2 kb and 2.5 kb fragments were visualized by autoradiography (Figure 4.9).



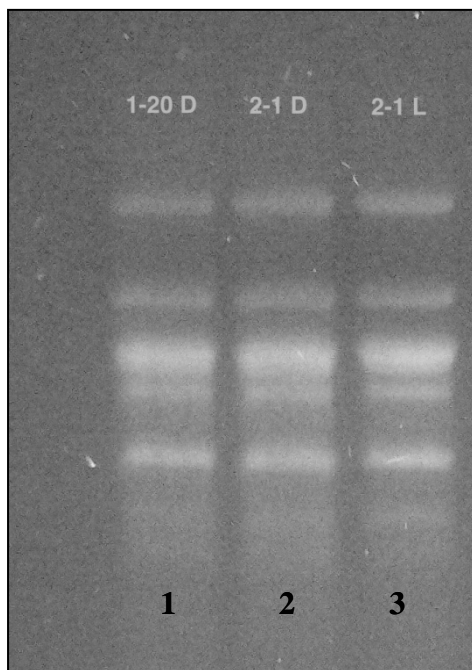
**Figure 4.9:** Southern blot autoradiogram of *ectA\_B* transformation vector and DNA from transformants digested with *KpnI* and *SnaBI* after hybridization to a radiolabeled *atpB* probe. The x-ray film was exposed for approximately 2 hours at -70 °C.

Due to a high amount of *ectA\_B* transformation vector loaded into the gel (Figure 4.8), a very strong signal is detected by the *atpB* probe in lane 1 on the membrane (Figure 4.9).

Using the 5.2 kb DNA fragment of the *ectA\_B* transformation vector, that hybridized strongly to the *atpB* probe, as a size marker for the hybridization signals of the two samples, it can be concluded that about 40% and 70% of the chloroplast genome copies carry the *ectA\_B* genes in transformant 1-20 and 2-1, respectively (Figure 4.9). This is in accordance with results from the slot blot, where transformant 2-1 gives a stronger signal.

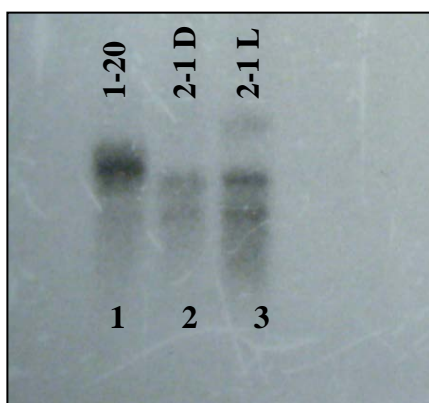
#### 4.4.2 Northern blot analysis

Expression of the *ectB* gene in transformants 1-20 and 2-1 was investigated by Northern analysis. Transformants were grown in 12h light/ 12h dark cycles and total RNA was isolated at 11 hours in the dark period and 1 hour in the light period. One of the samples, transformant 1-20 grown in light, was lost under the RNA isolating procedure. The RNA samples (4 µg) were separated on a formaldehyde-agarose gel (Figure 4.10) and transferred to a nylon membrane. After hybridization to a <sup>32</sup>P-radiolabeled *ectB* probe, the presence of *ectB* mRNA was visualized by autoradiography (Figure 4.11).



**Figure 4.10:** Agarose gel of RNA isolated from transformed cells in light and dark. Transformant 1-20 grown in the dark (lane 1), 2-1 grown in dark (lane 2) and 2-1 grown in light (lane 3).

Hybridization signals were weak, even after 3 days exposure, indicating either low rates of transcription or unstable transcripts (Figure 4.11). Moreover, *ectB* transcripts of correct size were only found in transformant 1-20 suggesting that the *ectB* construct in orientation 2 is not transcribed efficiently. In orientation 2 the *ectB* gene is located on the same DNA strand as the *ectA* gene and transcribed in the same direction (Figure 4.2) whereas in orientation 1 the two genes are located on different strands and transcribed divergently (Figure 4.1).



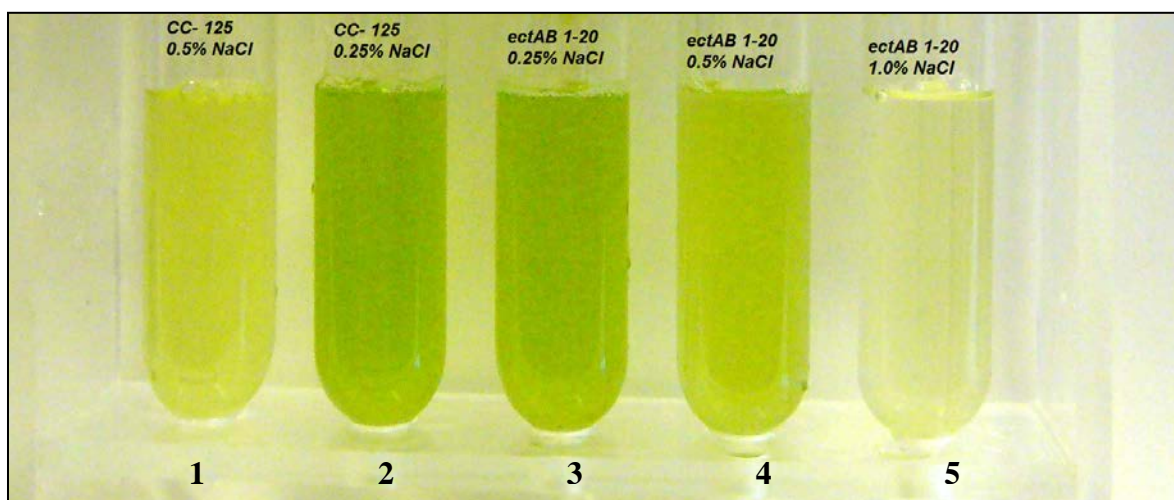
**Figure 4.11:** Autoradiogram of a Northern blot. Transformant 1-20 grown in dark (lane 1), transformant 2-1 grown in dark (lane 2) and transformant 2-1 grown in light (lane 3). Exposure of the x-ray film to the nylon membrane was 3 days.

For transformant 2-1 it appears that there are more *ectB* transcripts in light than in the dark but, because of the long exposure time of 3 days and because the size of the RNA is not as expected, further analyses will be needed to clarify these results.

### 4.4.3 Salt tolerance

*Chlamydomonas* transformant 1-20 was grown photosynthetically in HS medium and salt concentrations ranging from 0.25 - 1.0% NaCl (w/w), corresponding to 43 mM - 170 mM NaCl. Simultaneously, the wild type strain CC-125 was grown as a control in 0.25% and 0.5% NaCl (Figure 4.12). For comparison, sea water has an average salt concentration of 600 mM and salt-tolerant bacteria can grow at NaCl concentrations of 5 M.

No significant increase in salt tolerance of the transformant due to *ectA* and *ectB* gene integration was detectable. The higher the salt concentration the less growth is observed. A salt concentration of 1.0% prevented cell growth, indicating a low tolerance of *Chlamydomonas* towards salt.



**Figure 4.12:** *Chlamydomonas* cells grown phototrophically in varying salt concentrations to determine the salt tolerance in transformant 1-20. Wild type cells grown in 0.25% and 0.5% NaCl (tube 1 and 2). Transformant 1-20 grown at 0.25%, 0.5% and 1.0% NaCl (tube 3-5).

## 5 DISCUSSION

In this study the *ectB* gene from *Pseudomonas stutzeri* was stably inserted along with the *ectA* gene into the chloroplast genome of *Chlamydomonas*. Southern analysis showed that about 40% - 70% of the genome copies in the chloroplast of transformants harboured the *ectAB* construct. According to the Northern analysis, transcript levels depended on the orientation of the *ectB* gene relative to the *ectA* gene in the genome. Transcript accumulation was relatively low, particularly for *ectB* transcripts, suggesting either low rates of transcription or low transcript stability. Expression of the *ectA* and *ectB* genes did not confer any significant improvement in salt tolerance in *Chlamydomonas*.

### 5.1 *EctB* transcript levels

The *psbD*-*rbcL*\_pMK-RQ plasmid was designed to contain the *psbD* promoter and the *rbcL* 3' region in order to direct expression of the *ectB* gene. The *psbD* promoter has been shown to produce high levels of transgene expression in previous studies (Barnes et al., 2005).

However, in this study it seems that the *psbD* promoter was not able to direct expression of the *ectB* gene sufficiently (Figure 4.11). Other strong promoters or 3' regions can be used as long as they differ for *ectB* and *ectA* since identical flanking regions increase the probability of homologous recombination between the *ectA* and *ectB* genes which might eliminate one of the genes.

Regarding the 3' region, its identity probably has little influence on expression levels and protein accumulation (Barnes et al., 2005). The 3' region of the *rbcL* gene contains two tandem hairpins loops that are important for 3' end processing and prevent degradation of the RNA by ribonucleases (Stern et al., 2010).

Degradation could also have been responsible for low accumulation of *ectB* transcripts in *Chlamydomonas*. Chloroplast transcripts of *Chlamydomonas* normally have long half-lives, up to several hours. However, chimeric transcripts have been found to be degraded faster in light than endogenous transcripts. For example, the half-life of a chimeric *rbcL*-GUS transcript was 20 minutes in light compared to 5 hours in the dark (Salvador et al., 1993). Since a smear is seen in the autoradiogram of the Northern blot (Figure 4.11), degradation

might also have been the case for *ectB* transcripts, if they were susceptible to photo-degradation. According to the Northern blot analysis (Figure 4.11), transformants growing in light had higher levels of *ectB* transcripts than those under dark conditions. In general, transcripts of *Chlamydomonas* have enhanced stability in the dark, compensating for low transcription rates, compared to transcripts in cells grown under light conditions (Nickelsen, 1998).

Franklin et al. (2002) demonstrated that high cell density leads to reduced light intensity among cultured cells. Subsequently, Coragliotti et al. (2011) showed that heterologous transcript accumulation increased during growth at high cell density and conditions with low light intensity.

In conclusion, low levels of mRNA accumulation could be caused by parameters like growth conditions and molecular processes affecting the rate of transcription and degradation of recombinant mRNA.

## 5.2 Accumulation of EctB protein

Due to time limitations direct protein detection techniques were not performed in this study. Methods like mass spectrometry and western blots could have helped to find out whether EctB is present in the transformants. Unfortunately, however, no antibodies against proteins involved in ectoine synthesis are available.

For expression of endogenous chloroplast genes in *Chlamydomonas*, low transcript abundance does not seem to limit the expression of a protein (Eberhard et al., 2002). Whilst other reports, such as Barnes et al. (2005), have demonstrated that the level of expression of heterologous transgenes and mRNA accumulation correlates with protein accumulation. In our study low *ectB* transcript levels may have resulted in low abundance of EctB protein in the chloroplast. The stability of transgenic mRNAs and protein accumulation depends on the 5' UTR inserted upstream of the translation start site (Salvador et al., 2004).

The reasons for not achieving high levels of recombinant protein expression in the chloroplast might be several. Not all foreign proteins have the same expression levels, and reasons behind their low expression are not fully understood (Potvin & Zhang, 2010).

Rate of translation is the main determinant of protein accumulation (Coragliotti et al., 2011). One particular factor effecting translation is codon bias, since rare codons are often correlated with reduced translation of mRNA (Franklin et al., 2002). Codon optimization of the *ectB* gene was not done in this study because of the size of the *ectB* gene (~1.3 kb) which would have been expensive to codon-optimize. It is likely that EctB protein accumulated to lower levels than the codon optimized EctA protein previously expressed by Lunde (2012).

Degradation is another factor that affects the yield of recombinant proteins, as proteins undergo proteolysis in the chloroplast of *C. reinhardtii* (Surzycki et al., 2009). Chloroplast proteolytic pathways are limited and the chloroplast envelope protects foreign proteins against degradation by most cellular proteases (Faye and Daniell, 2006). In addition, nucleus-encoded proteins that interact with mRNAs in the chloroplast may stabilize or destabilize recombinant proteins (Stern et al., 2010).

### 5.3 Transformant analysis

During the screening for transformants, *ectA* and *ectB* genes were identified in the chloroplast of two cell lines (Figure 4.7). The Southern analysis indicated that transformant 2-1, with the *ectB* gene in orientation 2, had most copies of the *ectAB* genes. Transformant 1-20 with orientation 1 seems to have only 40%-50% of its chloroplast genomes transformed (Figure 4.9). However, Northern analysis showed that transformant 1-20 had higher levels of *ectB* mRNA than transformant 2-1 and was therefore the better transformant (Figure 4.11). A high copy number of the transgene in the chloroplast genome does not necessarily lead to higher levels of gene expression. Eberhard et al. (2002) demonstrated that by decreasing the number of chloroplast genome copies with 5-Fluoro-2'-deoxyuridine, the abundance of most chloroplast transcripts and the protein synthesis were not particularly affected as they were insensitive to changes in gene copy number in the chloroplast.

It is not clear why the orientation of the *ectB* gene relative to the *ectA* gene is critical for transcription. Most likely assembly of the transcription initiation complex or 3' processing of *ectB* transcript may be affected by the distance to the *ectA* gene. Thus, it may be interesting to also look at the accumulation of *ectA* transcripts in the two *ectAB* transformants.

## 5.4 Salt tolerance

To assess any improvement in salt tolerance of *ectAB* transformants compared to wild-type cells, the cells were grown at different NaCl concentrations, up to 1% NaCl [171 mM] (Figure 4.12). Although a slight improvement in cell growth is seen, it can be concluded that *ectAB* transformants show no measurable increase in salt tolerance. The most likely reason for this is lack of ectoine synthesis in transformants. Considering that the average concentration of NaCl in sea water is 3.5% (599 mM), *C. reinhardtii* appears to be very sensitive towards salt in the medium.

## 5.5 Ectoine accumulation in *Chlamydomonas*

Preliminary analyses of extracts of transformant cells by HPLC did not find any ectoine (Kathi Moritz, personal communication). Assuming that the *ectAB* construct is expressed in the *Chlamydomonas* transformants analyzed, it seems likely that the third gene of the ectoine synthesis pathway in *P. stutzeri*, the *ectC* gene, is also required for ectoine synthesis in *Chlamydomonas*. *EctC* codes for L-ectoine synthase catalyzing the final step in ectoine synthesis (Figure 1.1). It is therefore proposed that the *ectA* gene is essential for synthesizing ectoine along with *ectC*. EctB is a transaminase whose function may be replaced by other transaminases in the chloroplast (Uwe Klein, personal communication). The hypothesis that all three genes are needed for ectoine synthesis in the *Chlamydomonas* chloroplast can be confirmed by introducing an *ectABC* construct.

Stability of ectoine in the chloroplast may also be a factor that contributes to ectoine accumulation. If ectoine is synthesized but unstable, it will not accumulate significantly inside the cells. It is also possible that ectoine leaks out of the cells and is diluted in the medium. In this case cells will not become salt-tolerant despite ectoine synthesis.

Expression of ectoine genes in chloroplasts has the potential for producing good and stable recombinant proteins in large scale because of the many copies of chloroplast genomes compared to the nucleus. Even though yield of ectoine in *Chlamydomonas* may not be comparable with the high yield of ectoine produced in bacteria, it may be comparable to what is obtained in plants.

## 6 CONCLUSION

The *ectB* gene was successfully cloned into the chloroplast genome of *Chlamydomonas reinhardtii* in two different orientations. Low levels of *ectB* transcripts were observed in the *Chlamydomonas* cells. Ectoine did not accumulate in the *Chlamydomonas* cells, nor was any salt tolerance observed. This was supposedly due to non-optimized *ectA* and *ectB* genes and the absence of the *ectC* gene from the construct.



## 7 FUTURE PROSPECTS

The results of this study show that expression of the *ectA* and *ectB* genes from *P. stutzeri* is not sufficient for measurable ectoine synthesis in the *Chlamydomonas* chloroplast. A number of measures could be taken to improve ectoine synthesis and accumulation.

Obviously, what should be done first is to introduce the *ectC* gene in addition to the *ectAB* genes. This should result in at least detectable amounts of ectoine, unless other factors interfere, e.g. instability of transcripts.

In addition, it might be possible to enhance transcription and translation of the bacterial genes in *Chlamydomonas*. Different promoter regions may be used in the gene constructs, e.g. the *psbA* promoter that has been shown to direct high rates of transcription of foreign genes in other studies (Surzycki et al., 2009). Translation could be enhanced by optimization of codons in the *ectABC* genes. Codon usage is quite different in *P. stutzeri* and the *Chlamydomonas* chloroplast, with some of the bacterial codons used very seldom in *Chlamydomonas*. Finally, additional rounds of screening of transformants might help to find cell lines that harbour the foreign genes in all copies of the chloroplast genome, thus accumulating more transcripts and, perhaps, EctABC proteins.

Analysis of expression of the *ectABC* genes in *Chlamydomonas* would benefit from antibodies against the gene products. The presence and amount of the EctABC proteins could be determined and correlated with ectoine concentrations.

Using fusion proteins as a platform for producing recombinant proteins may also be worth trying, not only does the protein yield in some cases increase significantly but the protein can possibly be traceable. Muto et al. (2009) fused the luciferase protein with endogenous ribulose biphosphate carboxylase (RbcL) and increased accumulation of recombinant luciferase protein.

In the salt tolerance experiment the ionic effects of NaCl may inhibit growth and result in cell death. To address the problem, sugar alcohols like mannitol or sorbitol may be used as they do not have an ionic effect. Studies have shown that these sugars are better than NaCl, as

NaCl makes the cells clump together and coagulate in the medium (Uwe Klein, personal communication).

## Appendix I: *ectABC* genes in *Pseudomonas stutzeri*

Yellow: *ectA*

Grey: *ectB*

Green: *ectC*

CATATGCCTACCCTAAAAAGGAATTCAATCAACAACCCCAAAGGCATTGTTTTGAGTTTCCCCAC  
CGTAATGCTCCGTCGCCCAACCGACGGCGACGGTTACAACCTTCATCAGCTGGTGGCGCGCTGCC  
AGCCCCCTCGATACCAATTCGGTCTACTGCAACCTGCTGCAGTGTTCCGATTTCGCTGACACCGCC  
ATCGCCGCAGAGAACGCCCAAGGCGAGCTGGTCGGTTTTCATCTCGGGTTACCGCCCCCTTCGCG  
GCCGGACACGCTGTTCTGCTGCGCAGGTCGCCGTCGACAGTTTCGATGCGCGGTTCAGGGGCTGGCCC  
TGCGCATGCTGCTGGCACTGACCGCCCGGGTCGCTCGCGAGTACGGCGTGCGTTACATGGAAACC  
ACCATCTCGCCGGACAACGGGGCGTCACAGGCGCTGTTCAAGCGGGCCTTCGACCGCCTCGATGC  
CAACTGCACGACGCGCACGCTGTTTGCCCGCGACACGCATTTCCGCCGGTCAGCACGAGGACGAGG  
TGCTCTACCGCGCCCGGCCGTTTACCCTTTCCCATCTAGAAGAAGAGCTCAAGGAGCACGCATGA  
AAACTTTTGAAGTGAATGAATCCAGGGCTAGCAACTAGTGCCGTTCCCTTCCCCGTGGTCTTCAAG  
CAGGCCCAGGGCGCCGAAGTGGTCACTCAGGACGGCAAGCGCTACATCGACTTCCTCGCTGGTGC  
CGGCACGCTCAACTACGGGCACAACACCCGGTGCTCAAGCAGGCGCTGCTCGAGTACATCGAGA  
GCGACGGCATCACCCACGGCCTGGACATGTACACCGAAGCCAAGGAGCGTTTCCCTCGAAACCTTC  
AACCGGCTGATCCTCGAGCCGCGCGGCATGGGCGACTACCGCATGCAGTTCACCGGCCCGACCGG  
CACCAACGCGGTTCGAGGCGGCGATGAAGCTGGCGCGCAAGGTCACCGGGCGCAACAACATCATCA  
GTTTCACCAACGGCTTCCACGGCTGCAGCATTGGCGCGCTGGCCGCCACCGGCAACCAGCATCAC  
CGCGGCGGCTCCGGCATCGGCCTCACCGATGTCAGCCGCATGCCGTACGCCAACTATTTTCGGCGA  
CAAGACCAACACCATCGGCATGATGGACAAGCTGCTCTCCGACCCGTCCAGCGGGATCGACAAGC  
CCGCCGCGGTGATCGTCGAGGTGGTCCAGGGCGAAGGCGGTCTGAACACAGCATCGGCCGAGTGG  
ATGCGCAAGCTCGAGAAGCTCTGCCGCAAGCACGAGATGCTGCTGATCGTCGATGACATCCAGGC  
CGGCTGCGGCCGCACCGGGACTTTCTTCAGCTTCGAAGAGATGGGCATCCAGCCGGATATCGTCA  
CGCTGTCCAAGTCGCTGTCCGGCTACGGCCTGCCGTTTCGCCATGGTGTGCTGCGCCAAGAGCTG  
GACCAGTGGAAGCCCGGCGAACACAACGGCACCTTCCGCGGCAACAACCATGCATTCGTCACGGC  
GGCCGCGGCGGTTCGAGCACTTCTGGCAGAACGACGCGTTTCGCCAACAGCGTGAAGGCCAAGGGCA  
AGCGCATCGCCGACGGCATGCAGCGCATCATCCGTCGCCACGGCCCGGATTCGCTGTTCTCAAG  
GGCCGCGGGATGATGATCGGCATCAGCTGCCCCGATGGCGAGATTGCCGCCGAGTGTGCCGCCA  
CGCCTTCGAAAACGGCCTGGTGATCGAGACCAGCGGCGCCACAGCGAAGTGGTCAAGTGCCCTCT  
GCCCCGTGATCATCAGCGATGAGCAGATCGACCAGGCACTTCCATCCTCGACAAGGCCTTTGCC  
GCCGTGATGAGCGAGCAGACCGAGAACCAAGCTTCCTGAGGTATCCGCAATGATCGTCAGAACCC  
TCGCCGAGTGCGAAAAGACCGACCGCAAGGTCCACAGCCAGACCGGCACCTGGGACAGCACGCGC  
ATGCTGCTCAAGGACGACAAGGTGGGATTCTCCTTCCACATCACCACCATCTACGCCGGCAGCGA  
GACGCACATCCACTACCAGAACCCTTCGAGTCGGTGTACTGCATCAGCGGCAATGGCGAGATCG  
AAACCATCGAGCACCTGCTGCGCGGTGGCAGCGAAGACATGAAGCTGGCCTGCGTCTTCAACCCG  
CCGCTCAACGGGCGCGAAGTGATGACGAAAGCGGCGTCTATCCTCTGGAGGCCGAAACCGTCTG  
ATACCGGTTTAACCGGGGCGGCCACCGCGCCGCCCTGCCATTACAAGAAAGGAGGTAAG

## Appendix II: Sequencing result of insert with orientation 1 in the transformation vector

Green: *rbcL* 3'

Violet: *ectB*

Yellow: *psbD* 5'

Turquoise blue: *rbcL* 5'

Brown: *ectA*

Blue: *psbA*

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AATTCTTTTTATTTTTCATGATGTTTATGTGAATAGCATAAACATCGTTTTTATTTTTATGGTGTTTA
GGTTAAATACCTAAAGTCGACATGAAAACTTTGAAGTGAATGAATCCAGGGTTCGCAGCTACTGCCG
TTCCTTCCCCGTGGTCTTCAAGCAGGCCAGGGCGCCGAAGTGGTCACTCAGGACGGCAAGCGCTACA
TCGACTTCCTCGCTGGTGCCGGCAGCTCAACTACGGGCACAACCACCCGGTGCTCAAGCAGGCGCTG
CTCGAGTACATCGAGAGCGACGGCATCACCCACGGCCTGGACATGTACACCGAAGCCAAGGAGCGTTT
CCTCGAAACCTTCAACCGGCTGATCCTCGAGCCGCGCGGCATGGGCGACTACCGCATGCAGTTCACCG
GCCCCGACCGGCACCAACGCGGTCGAGGCGGCGATGAAGCTGGCGCGCAAGGTCACCGGACGCAACAAC
ATCATCAGCTTCACCAATGGCTTCCACGGCTGCAGCATTGGCGCGCTGGCCGCCACCGGCAACCAGCA
TCACCGTGGTGGTTTTCGGGCATCAGCCTCACCGATGTCAGCCGCATGCCGTACGCCAATTACTTTGGC
GACAGACCAACACCATCGGCATGATGGACAAGCTCTTGTCCGACCCATCCAGCGGTATCGACAGCCGG
CGGCGGTGATCGTCGAGTGTGCAGGCGAAGCGCCTGACACCGCGTCGCGATGATGCGCAGCTCGAGAG
CTCTGCGCAGCACGAATGCTGCTGATCGTCGACGACTCAAGCGCTGCCGCCGAACCGGGACTCTCAGC
TCGAAGAATGCATCAGCGAATCGCAGCGTGTCCAGTCTGTCCGGCTACGGCTGCGATATTGCAAGTC
GCTGTCCGGCTACGGCCTGCCGTTCCGCATGGTGTGCTGCGCCAAGAGCTGGACCAGTGGAAGCCCG
GCGAACACAACGGCACCTTCCGCGGCAACAACCATGCATTGTCACGGCGGCCGCGGCGGTTCGAGCAC
TTCTGGCAGAACGACGCGTTCGCCAACAGCGTGAAGGCCAAGGGCAAGCGCATCGCCGACGGCATGCA
GCGCATCATCCGTCGCCACGGCCCCGATTTCGCTGTTCTCAAGGGCCGCGGGATGATGATCGGCATCA
GCTGCCCCGATGGCGAGATTGCCGCCGAGTGTGCCGCCACGCCTTCGAAAACGGCCTGGTGATCGAG
ACCAGCGGCGCCACAGCGAAGTGGTCAAGTGCCCTCTGCCCGCTGATCATCAGCGATGAGCAGATCGA
CCAGGCACTTTCCATCCTCGACAAGGCCTTTGCCGCCGTGATGAGCGAGCAGACCGAGAACCAAGCTT
CCTGAGTCGACTAATAATAAAACCTTTTATTCATGGTTTATAATATAATAATTGTGATGACTATGCACA
AAGCAGTTCTAGTCCCATATATATAACTATATATAACCCGTTTAAAGATTTATTTAAAAATATGTGTG
TAAAAAATGCTTATTTTAAATTTTATTTTATATAAGTTATAATATTAAATACACAATGATTAAAATTA
AATAATAATAAATTTAACGTAACGATGAGTTGTTTTTTTATTTTGGAGATACACGCAATACATATTTAT
TTACATTGAAGAAAACCTGCTAGGATTTTATTAGACAGGCCTTTATATTAAATTTTAAACAACTTTTTA
AAATTTATGTAAATTTATATAATATATAAATCTTTATTTTTTTATTACATTTCGATCGTTTAAATACATG
AACAGAATAGTGATATGCCTACCCATAAAAGGAATTCAATCAACAACCCCAAAGGCATTGTTTTGAG
TTTCCCCACCGTAATGCTCCGTCGTCCAACCGACGGCGACGGTTACAACCTTCATCAGCTGGTGGCGC
GCTGCCAGCCCCTCGATACCAATTCGGTCTACTGCAACCTGCTGCAGTGTTCGATTTTCGCTGATACC
GCCATCGCCGCAGAGAACGCTCAAGGCGAGCTGGTTCGGTTTCATCTCGGGTTACCGCCCCCTTCGCG
GCCGGACACGCTGTTCTGCTGTCGAGGTCGCCGTCGACAGCTCGATGCGCGGTTCAGGGGCTGGCCCTGC
GCATGCTGCTGGCACTGACCGCCCGGGTCGCTCGCGAGTACGGCGTGCCTTACATGGAACACCACATC
TCGCCGGACAACGGGGCGTCACAGGCGCTGTTCAAGCGGGCCTTCGACCGCCTCGATGCCAACTGCAC
GACGCGCAGCTGTTCCGCCGCGACACGCATTTCCCGGTTCAGCACGAGGACGAGGTGCTCTACCGCG
CCGGCCCGTTACCGTTTCCCATCTAGAAGAAGAGCTCAAGGAGCACGCATGACGACTTCAGGTCGTT
TCGGTTAATGTAATGTACTTTATTTACTACCTCATCAGATCAGCATATACGTGCGACCCGATGTGGTA
CTTTTAAATCGGTTAGACATTTAGTAATATAAATGTCTAACCGATTTTATAATGCGTAAAAAACCTTA
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TTTCACTACTAATGAAGTCTTTAGATATATATTTATATTACAGATGATACAGTTAACAAGTTATTCCC  
ACTGTGGTTCTTAAAAAACACTTAAGTAAAAACACATAACTCCACGTAAGCGCATTTTCTTACAATCA  
AAGAATGCCAATTGTTCTTTGCTTGTTGTAAAAGAAAACTAGGCTTTTCTTTGAAAACATTTCAGC  
ATATTCAACAACCTTTTATAGATTGGTTTAGCGGATGATGGGACTCG

## Appendix III: Sequencing result of insert with orientation 2 in the transformation vector

Yellow: *psbD* 5'

Violet: *ectB*

Green: *rbcL* 3'

Turquoise blue: *rbcL* 5'

Brown: *ectA*

Blue: *psbA*

TCGACTAATAATAAAACCTTTATTCATGGTTTATAATATAATAATTGTGATGACTATGCACAAAGCAG  
TTCTAGTCCCATATATATAACTATATATAACCCGTTTAAAGATTTATTTAAAAATATGTGTGTAAAAA  
ATGCTTATTTTTTAATTTTATTTTATATAAGTTATAATATTAAATACACAATGATTAAAAATTAAATAAT  
AATAAATTTAACGTAACGATGAGTTGTTTTTTTATTTTGGAGATACACGCAATGAAAACCTTTGAACT  
GAATGAATCCAGGGTTCGCAGCTACTGCCGTTCCCTTCCCCGTGGTCTTCAAGCAGGCCAGGGCGCCG  
AACTGGTCACTCAGGACGGCAAGCGCTACATCGACTTCCTCGCTGGTGCCGGCAGCTCAACTACGGG  
CACAACCACCCGGTGCTCAAGCAGGCGCTGCTCGAGTACATCGAGAGCGACGGCATCACCCACGGCCT  
GGACATGTACACCGAAGCCAAGGAGCGTTTCCCTCGAAACCTTCAACCGGCTGATCCTCGAGCCGCGCG  
GCATGGGCGACTACCGCATGCAGTTCACCGGCCCGACCGGCACCAACGCGGTCGAGGCGGCGATGAAG  
CTGGCGCGCAAGGTCACCGGACGCAACAACATCATCAGCTTCACCAATGGCTTCCACGGCTGCAGCAT  
TGGCGCGCTGGCCGCCACCGGCAACCAGCATCACCGTGGTGGTTTCGGGCATCAGCCTCACCGATGTC  
AGCCGCATGCCGTACGCCAATTACTTTGGCGACAGACCAACACCATCGGCATGATGGACAAGCTCTTG  
TCCGACCCATCCAGCGGTATCGACAGCCGGCGGGTGATCGTCGAGTGTGCAGGCGAAGCGCCTGAC  
ACCGCGTCGCGATGATGCGCAGCTCGAGAGCTCTGCGCAGCACGAATGCTGCTGATCGTCGACGACTC  
AAGCGCTGCCGCCGAACCGGGACTCTCAGCTCGAAGAATGCATCAGCGAATCGCAGCGTGTCCAGTCC  
TGTCCGGCTACGGCTGCGATATTGCAAGTCGCTGTCCGGCTACGGCCTGCCGTTCCGCCATGGTGTTGC  
TGCGCCAAGAGCTGGACCAGTGGAAGCCCGGCGAACACAACGGCACCTTCCGCGGCAACAACCATGCA  
TTCGTACAGGCGGGCCGCGGGTTCGAGCACTTCTGGCAGAACGACGCGTTCGCCAACAGCGTGAAGGC  
CAAGGGCAAGCGCATCGCCGACGGCATGCAGCGCATCATCCGTCGCCACGGCCCGGATTTCGCTGTTCC  
TCAAGGGCCGCGGGATGATGATCGGCATCAGCTGCCCCGATGGCGAGATTGCCGCCGAGTGTGCCGC  
CACGCTTTCGAAAACGGCCTGGTGATCGAGACCAGCGGCGCCACAGCGAAGTGGTCAAGTGCCTCTG  
CCCGCTGATCATCAGCGATGAGCAGATCGACCAGGCACTTTCATCCTCGACAAGGCCTTTGCCGCCG  
TGATGAGCGAGCAGACCGAGAACCAAGCTTCCTGAGCAATCTTTATTTTTCATGATGTTATGTGA  
ATAGCAATAACATCGTTTATTTTATGTTTTCAGGTTAAATACCTAAAGTCGACTACATATTTAT  
TTACATTGAAGAAAACCTGCTAGGATTTTATTAGACAGGCCTTTATATTAAATTTTAAACAACTTTTTA  
AAATTTATGTAAATTTATATAATATATAAATCTTTATTTTTTATTACATTTCGATCGTTTAAATACATG  
AACAGAATAGTG CATATGCCTACCCTAAAAAGGAATTCAATCAACAACCCCAAAGGCATTGTTTTGAG  
TTTCCCCACCGTAATGCTCCGTCGTCCAACCGACGGCGACGGTTACAACCTTCATCAGCTGGTGGCGC  
GCTGCCAGCCCCTCGATACCAATTCCGTCTACTGCAACCTGCTGCAGTGTTCGATTTCGCTGATACC  
GCCATCGCCGCAGAGAACGCTCAAGGCGAGCTGGTTCGGTTTCATCTCGGGTTACCGCCCCCTTCGCG  
GCCGGACACGCTGTTCTGTCTGGCAGGTCGCCGTCGACAGCTCGATGCGCGGTCAGGGGCTGGCCCTGC  
GCATGCTGCTGGCACTGACCGCCCGGGTTCGCTCGCGAGTACGGCGTTCGTTACATGGAAACCACCATC  
TCGCCGACAACGGGGCGTCACAGGCGCTGTTCAAGCGGGCCTTCGACCGCCTCGATGCCAACTGCAC  
GACGCGCACGCTGTTCCGCCGCGACACGCATTTCCGCCGTCAGCACGAGGACGAGGTGCTCTACCGCG  
CCGGCCCGTTACCGTTTCCCATCTAGAAGAAGAGCTCAAGGAGCACGCATGACGACTTCAGGTCGTT  
TCGGTTAATGTAATGTACTTTATTTACTACCTCATCAGATCAGCATATACGTGCGACCCGATGTGGTA  
CTTTTAAATCGGTTAGACATTTAGTAATATAAATGTCTAACCGATTTTATAATGCGTAAAAAACCTTA

TTTCACTACTAATGAAGTCTTTAGATATATATTTATATTACAGATGATACAGTTAACAAGTTATTCCC  
ACTGTGGTTCTTAAAAACACTTAAGTAAAAACACATAACTCCACGTAAGCGCATTTTCTTACAATCA  
AAGAATGCCAATTGTTCTTTGCTTGTTGTAAAAGAAAACTAGGCTTTTCTTTGAAAACATTTCAGC  
ATATTCAACAACCTTTTATAGATTGGTTTAGCGGATGATGGGACTCG

## Appendix IV: Position of insertion of *ectAB* genes into the chloroplast genome of *C. reinhardtii*

Yellow: *Kpn*I sites

Turquoise blue: *Sna*BI site

Black box: insertion site of *ectAB* constructs

Red & italic: *atpB* gene sequence

ATGTTAATTTTTGTATATTTTAAAGAATTTTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATAAAAACCGTATTACTATTCGTATTA**GGTACC**CCATTATATATTATACAT  
TAAAAAAATAAAAAATTTTTTTTTTATTTTATTTTTTTTTTATTTTATTAATTATTTTATTAATT  
ATTTTATTAAATACCATGTGTGTATTCTAAATAGGTTTATTGGGGTACTTTATTTTACACTT  
TATTTTACACTTTATTTTACACTTTATTTTACACTTTATTTTACACTTTATTTTACACTTTA  
TTTTACACTTTATTTTACACTTTATTTTACACTTTATTTTACACTTTATTTTACACTTTATT  
TTACACTTTATTTTACACTTTATTTTACACTTTATTTTACACTTTATTTTACACTTTATTTT  
ACACTTTTTTAATATGAATTAATAATTAATTTAATTTAATTTAAGAGAACCAATTACTG  
GTATAATTTGGAAAATAAAATACATTTAATCTGGTAATTTATTTGGTAATTTGGTCAAAATT  
ATAATAGTTTTTATTTGATTTTTTTGAATTGAATTTAAAAAGACATCCCCTTACAGTATTCT  
TGCAAACCTTACGGGACAATAAAATAAATTTGTTCCTGCCAACTGCCGATATTTATATACTA  
GGACGTCCCCTTCCCCTTACGGGATATTTATATACTCCGAAGAGCAAGTAACTTAGGGATT  
TTAATGCAATAAAATAAATTTGTCCCCTTACGGGAATATAAATATTAGTGGACGTCCCCTTCG  
GGCAAATAAATTTTAGTGGCAGTTGCCTCGCCTATCGGCTAACAAGTTCCTTCGGAGTATAT  
AAATATAGGACGTCAGTGGCAGTTGCCTGCCAACTGCCTATATTTATATACTGCGATAAACT  
TTAGTTGCCGAAGGGGTTTACATACTCCGAAGGAGGAAGCAGGCAGTGGC**GGTACC**ACTGCC  
ACTAAAATTTATTTGCCTCCTAACGGAGCATTAAAATCCCGAAGGGGACGTCCCTGCCAACTG  
CCCTTGCCGCAGTATTAACATCCTATATTTATATACTCCGAAGTATATTTATATGCTTCCCC  
TTCCTTCGGGTATATAAATATTGGGCAAGTAACTTAGGAGTATATAAATATAGGACGCCAG  
TGGC**AGTGGTACC**GCC**ACTGACGTCCACTAATATTTATATTCCCGTAAGGGGACGTCCCTCCT**  
TCCCCTTCCCCTTCGGGACGTCCCTTCGGGTAAATAAATTTTAGTGAGTTGCCTGCCAACTGCC  
TAATATAAATATTGGGCAAGTAACTTAGCATGTTTACATACTCCGAAGGAGGACGCAGTGG  
CAGTGGTACAATAAATAAATTGTATGTAAACCAGTGCCAAGCATGTTTACATACTCCGAAGG  
ACGCCAGTGGAGTGGTACAATAAATAAATTTGTATGTAAACCTGCTACCGCAGCAAATAAATT  
TTATTCTATTTTAATACAATAAATAAATTTGTGGCAGGCAACAAATTTATTTATTGTCCGA  
AGGGGAAGGGGACGTCCCTGAAGGGAAGGTGCAACTACCTGGGAATTTTACCGATTAAATTA  
ACCTTTTAAATAATTTTTTAAAAATACTTTTTTAATACCTTGTTTTGTTCTTAAAGAACTG  
TATCTCGTGGACGACAAATATAATTTGGCGTTTTACACTCTTATTATTTACTCGCACGTTT  
CCTTTAGTTTTTTGCTGAAAAATAAAATTAGTATTTAACACATTATTAATAACAGTTTAA  
CGGCGTCCTTTATTTTAAAGTAAATAGATTAAATAGCTAATATGACATATATAATGTATGTG  
TAATTAATAAATAAATTGGCTCTTTAAGAAGAAAACAACCTAATGGTGTCCAAATATTTATTTT  
AATTACACATAAAAAATTAA**TTATTTTAATGAAGCAGCTTTACTAATAGCTTCTGTAATGT**  
**TACCTACTAAGTAGAATGCTTGTTCTGGTAAATCATCTAATTCACCAGCGAAAATTTTACCA**  
**AAACCTTCAATAGTTTCAGCTAAAGAAACATATTTACCAGGTGAACCTGTAAATACTTCAGC**



TACGAAGAATGGTTGTGATAAGAAACGTTCAATTTTACGAGCACGAGCTACAATAAGTCTGT  
CTTCTTCAGATAAATTCATCTAAACCTAGAATAGCAATAATATCTTGTAATTCCTTTGTAACGT  
TGTAAGTTTTTTTTTACGCTTTGTGCAGAATCATAGTGTTCACCAAGAATCCACGGTTG  
TAACATAGTTGAAGTTGATTCTAATGGGTCAACCGCAGGGTAAATACCTTTAGCAGCTAAGT  
TACGAGAAAGTACTGTAGTAGCATCTAAGTGAGCGAATGTTGTAGCAGGAGCAGGGTCAGTA  
AGGTCATCTGCAGGTACATATACAGCCTGAATCGATGTGATAGAACCATCTTTAGTTGAAGT  
AATACGTTCTTGAAGACCACCCATTTCTGTAGCTAAAGTAGGTTGGTAACCTACAGCTGATG  
GCATACGACCTAATAAAGCAGATACTTCAGCACCAGCTTGACGAAACGGAAAATGTTATCA  
ATGAAGAATAATACGTCTTGTTTGTAAACATCACGGAAATATTCAGCCATTGTTAAAGCTGT  
TAAAGCAACACGCATACGAGCACCTGGTGGTTCGTTTCATTTGACCGTATACAAGAGCTACTT  
TTGAATCAGATAAGTTCTTTTCAACAATAACACCAGATTCTTTTCATTTCTGTGTAAAGGTCG  
TTACCTTCACGTGTACGTTACCAACACCAGCAAATACAGAAACACCACCGTGTGCTTTAGC  
AATGTTGTTGATCAGTTCCATAATTAATAACTGTTTTGCCTACACCAGCACCACCGAAAAGAC  
CAATTTTACCACCACGACGGTATGGAGCTAAAAGGTCTACAACCTTAAATACCTGTTTCAAAA  
ATTGATAAACGAGTATCTAAATCAACGAAAGCAGGAGCTGTACGGTGAATTGGTAAAGTTTC  
TTCAACTTTAACATTACCCATGTTATCTACAGGTTACCTAGAACGTTGAAAATACGTCCTA  
AAGTTACTTTACCTACAGGAACACTTAACGGTTTACCAGTATCTACAACCTCCATACCACGC  
ATTAAACCTTCTGTTGGGTTCATAGAACTGCACGTACACAGTTATCACCTAAAAGTTGTTG  
AACTTCACAAGTAACAGCCATTTCTGTACCTGCTGAGTTTTTAGCACGAATAGTTAGAGCGT  
TGTAATATTTGGTACTTGGCCTTTAGCAAATACGATATCTAAAACAGGACCAATAATTTGT  
ACAATACGTCCCATGTTTTTTGTTTCAATAGAATCACTCATAGTAAGTGGAATTAATATGCC  
CCAAGGCATTCATATTTTAACTTATTTTACTTAAATTCTTACGTAATAACCCCGAAGGAAAC  
TTTATCTTAGGTAATTTGAAATAAGAACCTCCTCCTTCCCCTTCGGGCAAGTAAACTTAGCA  
TGTTTACATACTCCGAAGGAGGACGCCAGTGCGAGTGGTATCGCCACTGCCTAGTATGTAAA  
CATTGTGGCTTTACTTTCAAAAAGGCAGGAAATACTGAGAGTGTAACCTGTTATAAATTTAA  
AATTTATATAAAAAGGAAGCTCAAACATATAGTAGAATTTATATATAAAAAGTATTATTTCACT  
AACGCTTATTTTTTTAGTTTTTTTCATTTAACTATATATATTAATATATTATATAA

## Appendix V: Solutions and recipes

### **E. coli and DNA:**

#### **1% agarose [60mL]:**

Add 0.6 g agarose to 60 mL TAE buffer [1x]. Boil the solution in a microwave oven. Cool down to 50 °C with cold water before pouring into tray and placing the comb.

#### **Agarose gel loading buffer:**

0.25% bromphenol blue, 0.25% xylene cyanol FF and 30% glycerol.

#### **Ampicillin 60mg/mL (10mL):**

To 600 mg of ampicillin, add dH<sub>2</sub>O to a final volume of 10 mL. The solution is sterilized by filtering 1 mL solution to each 1.5 mL Eppendorf tube. Store the tubes at -20 °C.

#### **Kanamycin 50mg/mL (10mL):**

To 500 mg of kanamycin, add dH<sub>2</sub>O to a final volume of 10 mL. The solution is sterilized by filtering 1 mL solution to each 1.5 mL Eppendorf tube. Store the tubes at -20 °C.

#### **Lysogeny broth (LB) (1L):**

10g tryptone, 5g yeast extract, 10g NaCl, 200μL 5M NaOH. Add dH<sub>2</sub>O to 1L. Autoclave the solution at 121 °C for 20 minutes.

#### **Lysogeny broth (LB) with 1.5% agar (1L):**

10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar, 200μL 5M NaOH. Add dH<sub>2</sub>O water to 1 L. Autoclave the solution at 121 °C for 20 minutes.

#### **Potassium acetate (5M potassium, 3M acetate):**

294.42 g potassium acetate is added in 100 mL dH<sub>2</sub>O. Add glacial acetic acid until a pH of 4.6 is reached [ca. 40-50% of final volume]. Final volume should be 1 L.

#### **TEG [1L]:**

25mM Tris-HCl (pH 8.0), 10mM Na<sub>2</sub>-EDTA, 50mM glucose.

#### **TAE (Tris-acetate EDTA) buffer [50x]:**

242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0) in 1 L dH<sub>2</sub>O.

### **Chlamydomonas:**

#### **DNA extraction buffer:**

[100mM Tris (pH 8.0), 50mM Na<sub>2</sub>-EDTA, 0.5M NaCl, 10mM β-mercaptoethanol].

#### **High salt (HS) medium [1L]:**

20 mL salt stock [50x], 20 mL phosphate stock [50x], 1 mL trace elements. Add dH<sub>2</sub>O to 1 L, and autoclave for 20 minutes at 121 °C.

**High salt high acetate (HSHA) [1L]:**

20 mL salt stock [50x], 20 mL phosphate stock [50x], 1 mL trace elements, 2.5 g potassium acetate, bring the volume to 1 L by adding dH<sub>2</sub>O. Autoclave for 20 minutes at 121 °C.

**Hutner trace elements:**

Solution	Amount
Na <sub>2</sub> EDTA · 2H <sub>2</sub> O	5 g
ZnSO <sub>4</sub> · 7H <sub>2</sub> O	2.2 g
H <sub>3</sub> BO <sub>3</sub>	1.14 g
MnCl <sub>2</sub> · 4H <sub>2</sub> O	506 mg
FeSO <sub>4</sub> · 7H <sub>2</sub> O	499 mg
CoCl <sub>2</sub> · 6H <sub>2</sub> O	161 mg
CuSO <sub>4</sub> · 5H <sub>2</sub> O	157 mg
Mo <sub>7</sub> O <sub>24</sub> (NH <sub>4</sub> ) <sub>6</sub> · 4H <sub>2</sub> O	110 mg
Distilled water	100 mL

Adjust the pH to 6.5-6.8 with KOH (~1.6 g) at 70 °C. Store the solution in a refrigerator [5 °C]. The solution should be violet before use.

**Phosphate stock [50x] (500mL):**

47 g K<sub>2</sub>HPO<sub>4</sub> · 3 H<sub>2</sub>O (36 g water free), 18 g KH<sub>2</sub>PO<sub>4</sub>. Add dH<sub>2</sub>O to a final volume of 500mL.

**Salt stock [50x] (500 mL):**

12.50g NH<sub>4</sub>Cl, 0.5g MgSO<sub>4</sub> · 7H<sub>2</sub>O, and 0.25g CaCl<sub>2</sub> · 2H<sub>2</sub>O. Add dH<sub>2</sub>O to a final volume of 500mL.

**Analytical methods:*****Blotting:*****SSC [20x] (saline-sodium citrate):**

175.3 g NaCl [3M], 88.2 g Sodium citrate · 2H<sub>2</sub>O in dH<sub>2</sub>O[300mM]. Adjust pH to 7.0 with HCl solution and bring to 1 L.

**Labelling buffer:**

Make the following solutions:

- 1) 625 $\mu$ L Tris [1M] (pH 8.0), 62.5 $\mu$ L MgCl<sub>2</sub> [1 M], 8.7 $\mu$ L  $\beta$ -mercaptoethanol [14.4M] in 1mL.
- 2) 16.4g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in 25mL pH 6.6.
- 3) Random, primers (pd[N]6) (Pharmacia), 50 units/mL in TE (Tris [10mM], Na<sub>2</sub>-EDTA [1mM] pH 7.5.

Mix 475 $\mu$ L of solution 1, 500  $\mu$ L of solution 2, and 25  $\mu$ L of solution 3.

### ***Hybridization:***

#### **Hybridization buffer [100mL]:**

50mL Na-phosphate buffer [0.5M], 200 $\mu$ L EDTA [1mM], 1g BSA, 7g SDS in 100mL.  
Dissolve at room temperature.

#### **Na-phosphate buffer [1M pH 7.2] (hybridization):**

134g Na<sub>2</sub>HPO<sub>4</sub> · 7H<sub>2</sub>O, 4mL H<sub>3</sub>PO<sub>4</sub> [85%], add dH<sub>2</sub>O to 1L.

#### **Wash buffer #1 (1L):**

40mL Na-phosphate buffer [1M (pH 7.2)], 2mL EDTA [0.5M (pH 8.9)], 5g BSA, 50g SDS in a final volume of 1L.

#### **Wash buffer #2 (4L):**

160mL Na-phosphate buffer [1M (pH 7.2)], 8mL EDTA [0.5M (pH 8.9)], 40g SDS in a final volume of 4L.

### ***Southern blot:***

#### **DNA gel loading buffer:**

4% sucrose, 0.25% bromphenol blue.

#### **Neutralization solution:**

Tris [1.5M] pH 7.4, NaCl [1.5M].

#### **Denaturation solution:**

NaOH [0.5M], NaCl [1M]

### ***Northern blot:***

#### **MOPS buffer [5x]**

MgSO<sub>4</sub> [10mM], MOPS [0.5M] M, NaCl [2.5M]. Adjust pH to 7.5 with NaOH. Filter to sterilize and store the solution in the dark.

#### **RNA gel loading buffer:**

0.5mL glycerol [100%], 4 $\mu$ L Na<sub>2</sub>-EDTA [250mM (pH 8.0)], 2.5mg bromphenolblue, 2.5mg Xylene cyanol FF, and add sterile dH<sub>2</sub>O to 1mL. Treat with DEPC before use.

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